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Vol. LVII *November, 1929* *No. 5*

BIOLOGICAL BULLETIN

EGG LAYING HABITS OF *GONIONEMUS MURBACHII* IN RELATION TO LIGHT.[1]

MR. ROBERTS RUGH,

MARINE BIOLOGICAL LABORATORY AND COLUMBIA UNIVERSITY.

According to H. F. Perkins (*Proc. Philadelphia Acad. Sc.*, 1902) *Gonionemus murbachii* lays eggs only at night or in artificial darkness during the day and egg-laying can be induced more easily in the afternoon than earlier in the day. The tissues "get ready" for dehiscence of eggs within one minute after the animal is placed in the dark and the constancy of egg-laying is not affected by temperature.

It is the purpose of this paper to discuss some additional quantitative data which has been secured in regard to the egg-laying habits of *Gonionemus murbachii* and the effect of vital stains on these habits. The following data are to be used as control observations in an attempt to determine which part (or parts) of the visible spectrum is responsible for this spawning reaction. Report of these further experiments will follow.

METHOD

Gonionemus murbachii lays eggs which, very shortly after being shed, will sink to the bottom and by means of a sticky surface will adhere to any objects with which they come into contact. The eggs are shed by vigorous contractions of the bell (Perkins) and since the animal, by these contractions, stirs up the water in which the eggs are laid, even distribution of the eggs is insured.

Crystallizing dishes measuring 7½ cm. deep and 13.5 cm. in diameter and having a capacity of one liter were used. The bottom surface of these dishes was calculated to be 143 square centimeters. In each dish were placed three glass slides which had

[1] To Dr. C. G. Rogers of Oberlin I am indebted for the original introduction to the problems herein involved.

previously been marked off with a glass cutter into twelve square centimeters each. This gave a total of thirty-six square centimeters of bottom surface that were clearly indicated. The slides were so placed in the dishes as to be at right angles to each other and to form a wide figure "H." Careful examination of a number of dishes containing eggs was made and it was concluded that the average of the thirty-six square centimeters of bottom surface that was clearly marked off would give an accurate basis for estimaion of the total number of eggs on the 143 square centimeters of bottom surface.

The animals were collected between 8:30 and 11:30 A.M. and were used the same day. Only the largest individuals were chosen, the females being identified under the low power of the microscope. A female would be placed in a crystallizing dish to which had been added 850 cc. of fresh sea water, sea lettuce and scrapings of diatoms from eel grass (food). Water, slides, crystallizing dish, and food were changed for each female after each reading and a large photographic dark room was used for confinement. Each dish was covered by a glass plate to prevent evaporation and temperature readings were taken at the beginning and end of each designated period.

Data and Discussion.

The control individual (Female 7-10-*A*) was kept under observation for a period of eleven days during which time it laid 75,579 eggs, or an average of 6872 eggs per night. The extreme variations in number were 4,380 and 9,387, the higher number being laid toward the end of the observation period. This individual was exposed to natural light from 9:30 A.M. until darkness when it was placed in artificial light until 9:30 P.M. every night. From 9:30P.M. until 9:30 A.M. it was confined in the dark room. During the day it was placed for a very short period in direct sunlight. Fresh sea lettuce, sea water and food were placed in the dish daily, as was also the case with all of the experimental animals. This individual laid no eggs during exposure to light, whether daylight or artificial light, but laid eggs very consistently during the dark confinement at night

The case history of Female 7-10-*G* indicates several unique

situations and warrants a record of its history here. This female was watched for a period of fifteen days during which it laid 134,280 eggs or an average of 8,952 per day. After several days of observation, the individual was confined to the dark for a period of 44½ hours following which it was exposed to light for 13 hours and again confined to the dark. The result was that this female laid the greatest number of eggs in a single night recorded in the whole series of observations, namely 22,165 eggs. This may represent a "piling up" situation, due in part to long confinement in the dark followed by a normal period in light. The history reads as follows:

9:30 P.M. (D.)— 9:30 A.M.—10,560
9:30 A.M. (L.)— 9:30 P.M.—none
9:30 P.M. (D.)— 9:30 A.M.—11,466
9:30 A.M. (L.)—11:30 A.M.—none
11:30 A.M. (D.)— 7:00 P.M.—11,726
7:00 P.M. (L.)— 7:30 P.M.—none
7:30 P.M. (D.)— 8:30 A.M.—4719
8:30 A.M. (L.)— 9:30 A.M.—none
9:30 A.M. (D.)—11:30 A.M.—14,157
11:30 A.M. (D.)— 2:00 P.M.—none
2:00 P.M. (D.)— 5:00 P.M.—none
5:00 P.M. (D.)— 9:30 P.M.—none
9:30 P.M. (D.)— 8:30 A.M.—none
8:30 A.M. (L.)— 9:30 P.M.—none
9:30 P.M. (D.)— 9:30 A.M.—*22,165*
9:30 A.M. (L.)— 9:30 P.M.—none
9:30 P.M. (D.)— 9:30 A.M.—9004
9:30 A.M. (L.)— 9:30 P.M.—none
9:30 P.M. (D.)— 9:30 A.M.—5720, etc.

One further case may be reported here as being an exception to the general summary of the paper, though the "exception" can be adequately explained. This is the only case among the thirty-nine histories recorded in which any eggs were laid in the light. It seems probable that this was due to a sudden interruption of the normal egg laying period by bringing the individual into the light half an hour after having been placed in the dark. The eggs

that were laid probably represented those which would have been shed with the next several contractions of the bell. This contention is somewhat supported by the fact that the number of eggs shed is relatively very small. In any case, this seems to indicate that all of the eggs are not laid immediately upon confinement in the dark, as was suggested by Dr. Perkins.

Female 7-17-*O*

9:30 P.M. (D.)— 9:30 A.M.—5722
9:30 A.M. (L.)— 9:30 P.M.—none
9:30 P.M. (D.)—10:00 P.M.—4576
10:00 P.M. (L.)— 9:30 A.M.—1431
9:30 A.M. (L.)— 9:30 P.M.—none
9:30 P.M. (D.)— 9:30 A.M.—8500, etc.

Summary and Conclusions.

1. *Gonionemus murbachii* never lays eggs in the light under normal environmental conditions.

2. The only occasion upon which eggs can be induced in the light is when the active egg-laying period (during the hour after confinement in the dark) is suddenly interrupted. Apparently the animal cannot interrupt the shedding of eggs that have been partly extruded, and the "irritation" of exposure to light probably causes the contractions of the bell to be somewhat more vigorous.

3. Eggs are never laid in the light even after prolonged exposure to light during the normal egg-laying season.

4. It is quite possible that *G. murbachii* in its normal habitat lays eggs during dusk rather than in total darkness and not in merely a few contractions of the bell as suggested by Perkins Natural darkness is, of course, gradual, while the experimental confinement in darkness is sudden.

5. Light is probably necessary for the maturation of the egg cells of *G. murbachii.*

6. Eggs will always be laid at night except under the following conditions:

(*a*) When exposed at night to artificial light.
(*b*) When kept in darkness since the preceding egg laying period.
(*c*) When placed in darkness for one hour or more during the

preceding afternoon. In this case eggs may be laid during the night but will represent the balance between the number laid in the afternoon and the number that would normally have been laid during the night.

7. At the height of the egg laying period there is a definite number of eggs that, under normal conditions, are ready for dehiscence every night. Some histories showed a variation upward in number of eggs laid during consecutive nights. These eggs may be laid:

(*a*) During the first hour of darkness following exposure to light (daylight or artificial light).

(*b*) During the afternoon in artificial darkness providing the eggs have not been shed too recently before confinement *or* the animal has been exposed to at least an hour of light preceding the confinement.

(*c*) During the morning in artificial darkness if the previous night's supply has been held back, entirely or in part, by exposure of the animals to light during the night.

8. There is a physiological limit to the number of mature eggs which can be accumulated in light for a particular animal. There is, therefore, no correlation between the length of light exposure and the number of eggs matured.

9. The animals do not measure over 2½ centimeters in diameter, hence the maximum of 22,165 is an almost incredible number of eggs for an individual to shed during one hour of confinement in the dark. An individual with six rays (gonads) laid fewer eggs than most of the four-rayed individuals, but there may have been undetectable maturity differences.

10. Thirty-nine *Gonionemi* representing 127 nights laid a total of 843,510 eggs or an average of 6,642 eggs per night per single jellyfish. These thirty-nine individuals had been selected as being relatively mature females.

11. Slight variations in temperature and pH which occur under normal experimental conditions have no correlation whatever with the numbers of eggs layed.

12. The commonly used vital stains, methylene blue, and neutral red, have, at the most, only a transient effect upon the number of eggs laid. If the number is reduced immediately after

staining it is made up during the subsequent egg laying period. There is the possibility that these vital stains may have some stimulating effect on the egg laying process. (A report is elsewhere to appear giving data to indicate that these vital stains increase the vitality and prolong the life of specimens of *G. murbachii*.) The stain may in some way cut out that part of the visible spectrum which inhibits normal egg laying.

The above observations indicate that there is a definite correlation between the egg laying habits of *Gonionemus murbachii* and light, whether the light is natural or artificial. Vital stains have at the most only a transitory effect upon these regular egg laying habits and these effects may be due in part to the light transmitting qualities of these stains. This leads us to the proposition that further experiments are in order which will determine the effects of various parts of the visible spectrum on these egg laying habits as well as the effects of other factors such as food variations and presence of the male. A few casual observations indicated that presence of the male was not sufficient to induce egg laying in light, or even to increase markedly the number of eggs laid. It is hoped that these further experiments will soon follow.

SOME OBSERVATIONS ON SPERM DIMORPHISM.

GERARD L. MOENCH, M.D., F.A.C.S., AND HELEN HOLT, B.S.

In recent investigations[1] of the fertility of about 150 men we studied first the morphology of the semen, and then calibrated accurately in 0.50 mm. the head lengths of the spermatozoa. The technique was the usual one for such studies; projection onto a screen of the image of the cell at a known definite magnification which was 3,000 diameters in our work. Such studies have been made before, but previous to the work of Williams and Savage (1–5 inclusive), measurements of the head lengths of mammalian spermatozoa were mostly only concerned with the question of dimorphism, and a certain large number of head lengths accurately calibrated at a sufficient magnification when arranged in a frequency distribution polygon were supposed to result in a dimodal graph. Thus Wodsedalek (6, 7) claimed such dimodalism resulted from measuring the sperm head length of the pig, horse, and bull. Zeleny and Faust (8) obtained similar results in the case of the ram, dog, and bull, and Parkes (9) in man, the rat and mouse, and perhaps the cat. It was therefore thought that two types of spermatozoa existed, one producing female and the other male offspring, as the difference in size of the two groups was explained by the presence or absence of the odd or sex-determining chromosome. However, Hance (10), working with the pig, has shown that the chromosomes, forty in number, are constant, but may vary as much as 12 per cent. in the spermatogonal pairs. Any definite division into group sizes on the basis of this finding must, however, be purely accidental. Williams and Savage (1, 2, 3), in their work with bull sperms, never found any dimodalism in normal animals. On the contrary, the closer the frequency polygon approached a normal frequency distribution, the better in most cases the reproductive fitness of the animal turned out to be—the cœfficient of variability especially being that function of the frequency distribution of the population

[1] With financial assistance from the New York Committee of Maternal Health.

which formed an indicator as to the animal's fertility. Thus, the fertility decreased as the cœfficient of variability increased. No semen specimen, of course, shows all the sperms to be of the same size, any more than all the cells are ever morphologically perfect. At the same time, Williams and Savage found that the sperm

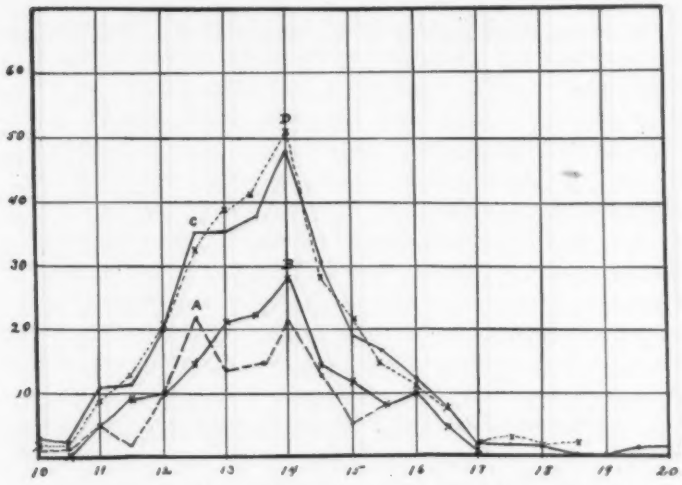

Fig. 1. Case No. 13, *B*. This case represents two graphs *C* and *D* from calibrations of 300 cells each on the same specimen, and, except for some minor changes, the two frequency polyhedrons have approximately the same shape and mathematically it can be shown that the differences between the two curves as compared to the probability of error is not significant and not greater than may be expected from random sampling. Repeated graphs of this kind were frequently made to determine the accuracy of our methods and to compare the accuracy of various methods of calibration. *A* represents first 131 cells of graph *C*; *B* second 169 cells of graph *C*; *C* equals combination of *A* and *B*. See also text.

In this graph, as in the other one here reproduced, the figures on the ordinate or vertical line give the number of heads of any particular length observed, whereas the figures on the abscissa, or horizontal line, give the size of the heads in millimeters (and half millimeters) at a magnification of 3,000 diameters.

heads from one ejaculate did not vary beyond reasonable limits in normal cases.

In our work with human spermatozoa we can to date but corroborate the conclusions of Williams and Savage. Never, even in our abnormal cases, did we see any evidence of dimorphism of the sperm head, although we were constantly on the lookout for it. The nearest approach to a dimodal graph obtained in our series of

cases in shown in Fig. 1. Here the first 131 cell heads measured gave a distinctly dimodal graph (Curve *A*), yet the curve straightened out in the next 169 cells, or for the total of 300 cells usually measured on each specimen.

The failure of Williams and Savage and ourselves to find sperm dimorphism in the bull and man, where it had been described before by others, must have some explanation. Perhaps some of the discrepancy present may be explained by the unit of measurement employed. Some observers used 0.25 mm. as a class unit, whereas both Williams and Savage and we used 0.50 mm. Since bull sperms are about twice as large as human sperms, 0.50 in our work really represents twice as coarse a scale, so that at first when trying out various methods to determine the best way to calibrate human sperm heads we also used a 0.25 mm. scale on a number of cases. We soon found out, however, that this class unit was so small, and the resulting minute differences of measurement so hard to judge that the results, instead of being more accurate, became less so. As a result, some of the cases calibrated with this unit of measurement showed an apparent sperm dimorphism-which, however, was not really present, since it disappeared when we re-measured these same specimens with a 0.5 mm. scale, It might seem to some, therefore, that our scale of 0.5 mm. was too coarse to show up dimorphism. This, however, must be denied, since the claimed differences in size of the two groups of sperm heads was always a number of times larger than our class unit. Hence it would seem to us, though it may sound temerarious to say so, that some of the reported instances of sperm head dimorphism were due to errors in the methods employed. This opinion would seem to receive corroboration from the work of Lush (11), who found much less dimorphism of the boar sperms than was claimed by Wodsedalek (6, 7). We have also had occasion to measure the sperm head lengths of the boar, and Fig. 2. shows the graph obtained by measuring 300 heads of a normal fertile boar. The beautiful symmetrical and normal distribution is at once apparent in this figure. Of course one positive observation is not contraindicated by any number of negative findings, still the hundreds of cases examined by Williams and Savage and ourselves presenting no sperm dimorphism offer strong evidence

that such dimorphism must at least be the exception rather than the rule. It must also be remembered that the material used by the various workers in this field was not always the same. While Williams and we employed ejaculated sperm cells, Wodsedalek

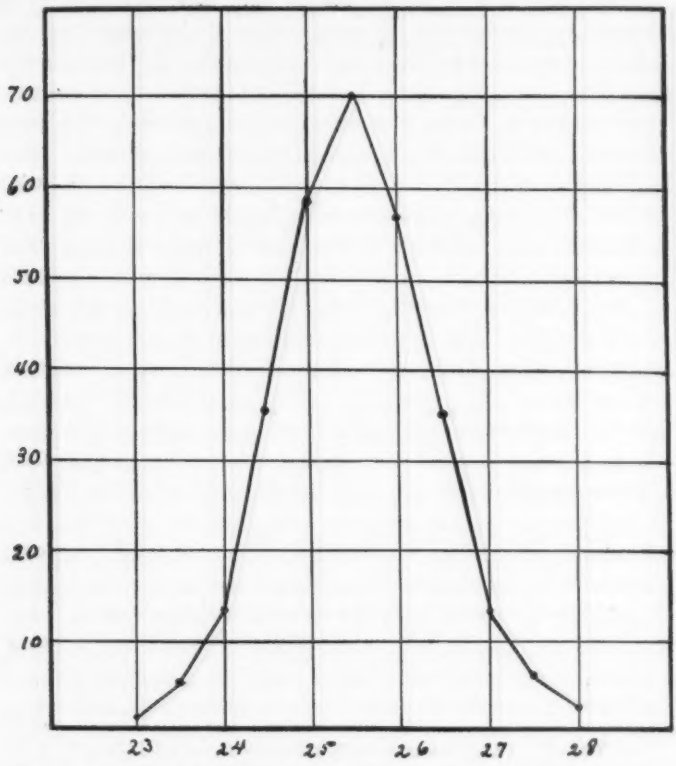

Fig. 2. This graph is the result of measuring 300 sperm head lengths of a known normally fertile boar.

made sections of the testicle and measured those spermatozoa which were free in the lumina of the tubules, since he considered these cells mature. It has been pointed out before that a number of observers believe that the sperms first mature in the epididymis. Furthermore, histological sections have gone through so many processes that one cannot tell how the spermatozoa may have

been affected. For this reason the claims set up for sperm dimorphism by Wodsedalek are at least subject to grave doubts, and any size class differences found may have been produced by pure accident.

The results obtained by Zeleny and Faust also seem to us fortuitous, and are not comparable to our investigations, since these observers measured only the basal, usually darker stained portion of the cell, as this only was supposed to represent the nuclear material. With the ever-increasing proof that the whole sperm head consists of nuclear material, Zeleny and Faust's work loses in importance, since no reason seems assignable why the length of the anterior, lighter portion of the sperm head probably representing the head cap should influence in any way the fertilizing power of the cell.

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STUDIES ON THE PANCREATIC SECRETION IN SKATES.

B. P. BABKIN.

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From the point of view of comparative physiology the skate possesses many features of interest. Animals such as the elasmobranch fishes, which are generally considered to have remained at a lower point of evolution than mammals, present an opportunity of investigating the intermediate stages of functional development of the different organs of the higher forms. An attempt was made in the present study to investigate the pancreatic secretion in skates, since the anatomical relation of the pancreatic gland in these animals affords certain advantages for such experimental study. In addition some observations were made on the distribution and relations of the pancreatic ducts which present certain peculiarities in this animal form.

Anatomical Data.

The pancreatic gland was investigated in three species of skates, namely, *Raja erinacea*, *Raja diaphanes* and *Raja stabuliforis*. The size of the organ varies of course according to the proportions of the animal. In all these the gland is quite compact, showing no tendency to take the diffuse form seen in most of the higher orders of fishes but rather resembling the analogous structure in the higher vertebrates, including man. Indeed the gland is much more compact in the skate than in the rodent type of mammalians. Disregarding minor differences in the shape of the pancreas in the three species examined, one finds that it always consists of two lobes of unequal size connected by a more or less constricted isthmus of pancreatic tissue. The ventral lobe, which is proximal to the intestine, is considerably smaller and lies attached to the groove formed by the junction of the duodenum and the pyloric end of the stomach. The dorsal lobe is much

larger and lies under the stomach. In the excised pancreas it flattens to a certain degree (Fig. 1).

For studying the distribution of the duct system, the ducts

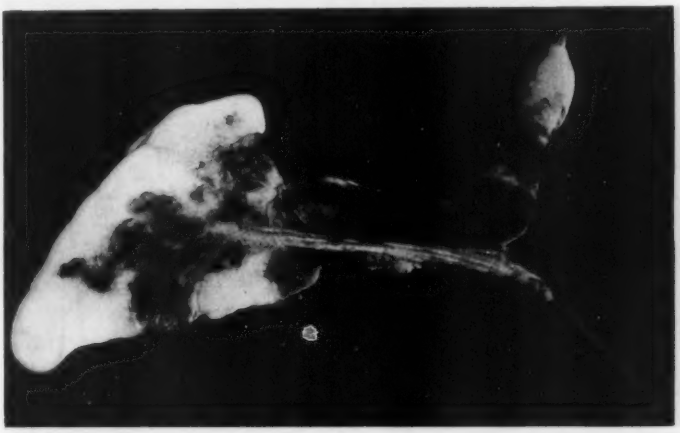

FIG. 1. Pancreatic gland of *R. stabuliforis* injected with carmin-starch-formalin mixture.

were in some cases injected with a mixture of carmin and starch in 10 per cent. formalin, and the fixed preparation was then carefully dissected out. In other cases the ducts were injected with pyroxylin dissolved in acetone and coloured with ultramarine-blue or vermillion. A cast of the duct system was then obtained by digesting away the surrounding glandular tissue by means of a mixture of pepsin and hydrochloric acid. At the Biological Station the mixture was made up by adding a quantity of 0.36 per cent. hydrochloric acid to the gastric mucous membrane excised from skates.

There is usually only one duct connecting the gland with the duodenum. This duct takes the form of a comparatively large tube of approximately uniform caliber, from which smaller ducts are given off. The large duct traverses the dorsal side of the isthmus of the gland and disappears into the midst of the distal lobe. Fig. 1 is a photograph of the pancreatic gland of *Raja stabuliforis* seen from the dorsal side and showing the main duct injected. The length of the fish was 121 cm. The length of the

visible portion of the main duct was 5.8 cm., while the portion connecting the proximal lobe with the duodenum measured 1.8 cm. The diameter of the visible portion of the duct was about 3.5 mm., but it became slightly reduced toward the point where the duct disappeared into the substance of the distal lobe of the gland. Figs. 2*a* and 2*b* are sketches of the pancreatic ducts of two specimens of *Raja stabuliforis* as they appeared after being injected with the mixture of carmin, starch and formalin. Fig. 3 shows a

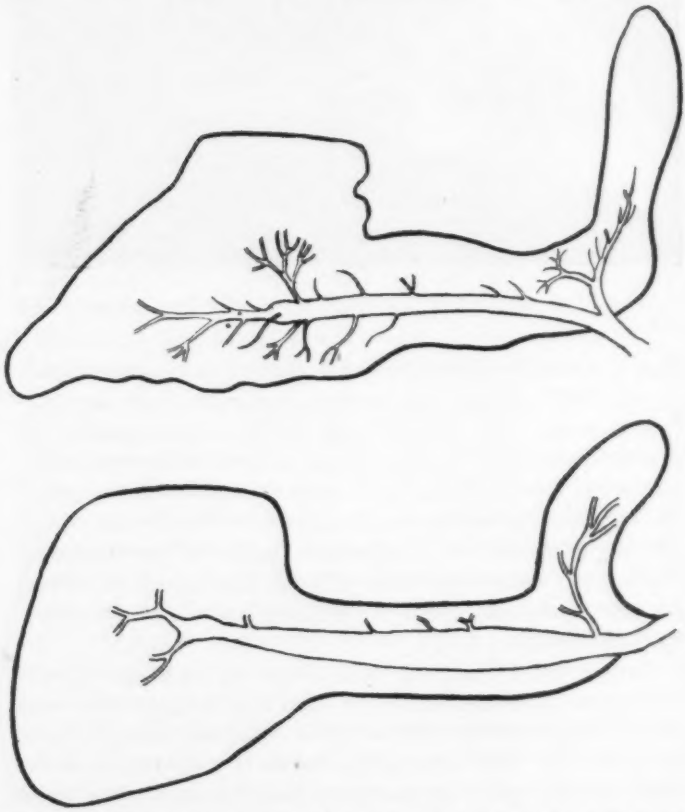

FIG. 2, *a* AND *b*. Sketch of pancreatic ducts of two *R. stabuliforis* injected with carmin-starch-formalin mixture. Only a few secondary, tertiary, etc. ducts could be injected. In case "*a*" the main duct presented a large tube of uniform size. In case "*b*" the main duct was dilated in the middle part.

similar preparation of *Raja diaphanes* and demonstrates the analogous structure of the gland in these two species.

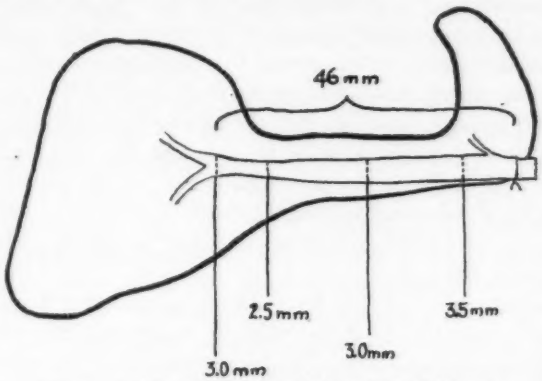

FIG. 3. Sketch of the pancreatic ducts in *R. diaphanes*.

The most striking feature of the duct system in the pancreas of the skate family is the contrast between the calibre of the main duct and that of the secondary ducts. In *Raja stabuliforis*, for instance, the only branch having a fairly large diameter is that which drains the proximal lobe of the gland. All other branches in this species have such extremely small channels that only the very beginning of each could be injected. In *Raja diaphanes* it was found to be very difficult to inject any of the secondary ducts, while in the case of *Raja erinacea* attempts to inject these ducts were entirely unsuccessful.

This peculiar contrast in calibre between the main and the secondary ducts suggests that the latter do not develop as fully in the skate as in higher forms. In mammals we find a gradual diminution in caliber, proceeding from the main duct or ducts through successively smaller divisions until the terminal ductules are reached. In the cat and dog, for instance, even the terminal ductules are of such a caliber that it is comparatively easy to inject the entire system of ducts, as Revell (1) has shown for the pancreas of the dog. On injecting the pancreas of the cat, the writer has observed very thorough penetration of the injection mass into the smallest ductules, but a similar injection could not be made to penetrate the secondary ducts of skates to any extent,

or when it penetrated the cast was so fine that the ducts could not be preserved intact during digestion of the pancreatic tissue.

It is interesting to note that in skates the "Langerhans cells" do not form the regular islands of Langerhans as in mammals. According to Jackson (2), most of the "Langerhans cells" in the pancreatic gland of skates remain in contact with the ducts. They are usually found between the cells forming the outer layer of the ducts. Jackson looks on this peculiar distribution of "Langerhans cells" in the skate's pancreas "not as constituting a fundamental difference as compared with other groups (*e.g.* mammals) but as a more primitive condition, of phylogenetic and ontogenetic interest."

It seems that the whole structure of the pancreas has a more primitive character in skates than in mammals. We are now engaged on a special histological investigation of the pancreatic ducts in the skate. It may be added that the pancreatic ducts are also extremely narrow in some bony fishes, and it is almost impossible to inject them, (Legouis (3), Krüger (4)).

Methods.

Two methods of immobilizing the animals were employed in the present investigation, namely, section of the spinal cord below the medulla and intraperitoneal injection of Dial "Ciba." The first method was based on the investigations of Miss Craw (5), who showed that spinal skates can live for a long time under proper conditions. The operation was performed as follows: A specimen was removed from the water, and its spinal cord was cut quickly below the medulla. A glass tube connected with the sea-water pipe system was inserted into one of the spiracles. If the section of the spinal cord was performed not too near the medulla oblongata respiratory movement continued for several hours. After section of the spinal cord the animal was turned on its back and the abdomen opened. A cannula connected with a graduated tube was then inserted into the pancreatic duct. The common bile duct was ligatured near the duodenum and a cannula connected with a graduated tube was fixed into the gall bladder. Through an opening in the pyloric part of the stomach a glass cannula (for injections into the duodenum) was inserted into the duodenum and tied. In some of the experiments, to prevent fill-

ing of the stomach with sea water, which runs through the spiracles and is sometimes swallowed by the animal, the œsophagus was tied near the cardia. In many of the experiments the rectum was also tied to prevent the escape of the solutions introduced into the duodenum.

In the case of immobilization by "Dial" Ciba (Basel) the animal was taken from the water, placed on its back and held by a wire-net with loops big enough to pass through a hypodermic needle. An injection was then made into the abdominal cavity by means of a syringe with "Dial" 0.35 to 0.40 c. cm. per kilo weight, and the animal put back in the water. In about a quarter of an hour the animal was asleep and underwent the operation described above. The dose of Dial required was much smaller than the corresponding dose for warm-blooded animals (*e.g.* 0.7 c.cm. per kilo weight for a dog or cat). Notwithstanding the skates were perfectly anæsthetized for 24 hours or more with this amount of Dial. Larger doses of Dial stopped the breathing, though it could usually be restored by means of artificial respiration.

The reaction of the pancreatic juice, bile and gastric contents was tested with litmus paper, and whenever possible the hydrogen ion concentration was determined by the colorimetric method.

The proteolytic activity of the pancreatic juice was determined by means of digestion of fresh calf's fibrin, preserved in glycerin and washed thoroughly before the experiment with running water. Protrypsin was activated by an extract of the intestinal mucous membrane of the skate.

The diastatic power of the juice was tested with 1 per cent. soluble starch solution (iodine and Fehling's tests).

The lipolytic power of the juice was determined according to the method of Anrep, Lush and Palmer (6).

The methods of preparation of the different extracts for enzyme determinations will be described later.

Elasticity and contractility of the Main Pancreatic Duct.

Before presenting data concerning the pancreatic secretion the properties of elasticity and contractility of the main pancreatic

duct will be discussed. These properties of the duct determined some special measures employed during the collection of the pancreatic juice. It could be seen very often in the course of an experiment that the fluid in the graduated tube instead of moving forward moved backwards a few divisions. Slight massage of the duct propelled the juice along the tube.

This backward movement of the fluid may sometimes be observed at the beginning of an experiment in spinal animals in quite good condition, but it is seen very often at the end of an experiment when the animal is dying or even shortly after death. Cutting the spinal cord too close to the medulla, which is usually followed by difficulty in respiration, has the same effect. In skates anæsthetized with Dial the contraction of the duct was not at all marked. Thus the main duct possesses a tone of its own, which may be increased or diminished under certain conditions. That we are not dealing with mere elasticity of the walls is shown by the following experiment:

Exp. Aug. 12. R. diaphanes. Spinal preparation. The pancreatic cannula and the graduated tube were filled with filtered sea water containing 0.5 per cent. of urea. The freezing point of this fluid was equal to $-1.89°$ C. The $\triangle$ of the blood of *R. diaphanes* is equal to $-1.80°$ C. (For these determinations I am indebted to Mr. A. F. Chaisson, who worked at the St. Andrews Biological Station.) This fluid was an indifferent one for the tissue of the skate.

From 10 A.M. to 10:30 A.M. the fluid moved from division 139 to division 140 of the graduated tube (1 division). At 10:30 A.M. the graduated tube was turned upright, and the level of the fluid sank very rapidly to 107 (33 divisions). The graduated tube was then closed and the rubber tube connecting it with the pancreatic cannula was twice gently compressed. As a result of this more fluid entered the gland, so that the fluid in the cannula moved up 36 divisions and now stood at 71. When the graduated tube was opened the fluid rose in it to 79 (8 divisions) and in five minutes fell again to 75 (4 divisions). Fifteen minutes later the level was at 74, and some fifteen minutes after that at 73. When the tube was placed horizontally, the fluid moved along it 25 divisions *i.e.*, reached the 98th division.

It may be seen from this experiment that sudden distention of the duct stimulated it to contract. Later it relaxed. When the pressure on the walls of the graduated tube was diminished by placing it horizontally, the fluid moved along 25 divisions. This phenomenon must be ascribed to the elasticity of the duct. Some of the fluid pressed into the gland did not return, being absorbed, or as seems more probable, remaining in the small ducts or in the interstitial tissue.

The preliminary histological investigation of the pancreatic gland in skates, performed in our laboratory by Dr. D. J. Bowie, showed that in the vicinity of the main pancreatic duct there are smooth muscular fibers.

In connection with these findings a method of very gentle pressure on the main duct during the secretory periods was adopted. This ensured that all pancreatic juice secreted during a certain time passed into the cannula and the graduated tube.

Pancreatic Secretion.

A spontaneous pancreatic secretion was noticed in almost every case. In the experiments the secretion in animals with an empty stomach and duodenum was very scanty. In *R. diaphanes* it averaged only 0.02 c.cm. in one hour. Sullivan (7) has attempted to collect the pancreatic juice in *Carcharias littoralis* over several days. Through an incision in the abdomen a glass cannula was inserted and fastened in the central end of the pancreatic duct. To the outer end of the cannula a small sterilized balloon was attached. Although there was great difficulty in keeping such fish alive, the operation was successful in six cases. The quantity of juice thus collected by Sullivan was as a rule small, however, and it had no digestive activity.

The narrowness and fragility of the pancreatic duct in *Scyllium catulus* and *Lamna cornubica* according to Yung (8) make the preparation of a fistula in the living animals impossible.

Attempts were made to stimulate pancreatic secretion by the following methods: (1) Introduction into the duodenum of HCl solution of different concentrations (0.36 per cent. to 0.95 per cent.). (2) Introduction of a mixture of equal parts of 0.36 per cent. HCl + 2 per cent. urea solution. (3) Intravenous injection of secretin (a cannula was inserted in the central end of one of the gastric veins; secretin was prepared in the usual manner by the action of 0.36 per cent. HCl on the mucous membrane of the duodenum and of the spiral intestine of the skate). (4) Injection of pilocarpin hydrochloride solution intravascularly and into the ducts of the pancreas.

The introduction of HCl solution into the duodenum usually had a definite positive secretory effect. 0.49 per cent. HCl solution was more effective than 0.36 per cent. and 0.96 per cent. solutions.

In some cases the action of the hydrochloric acid was very insignificant. This was usually the case in fasting animals. On the other hand, the introduction of an acidified peptone solution or of an acid digest of fish muscle increased the subsequent secretory effects of HCl solution. In the experiment quoted below (Experiment of July 23) 0.49 per cent. HCl injected into the duodenum after an acid digest, gave the greatest secretion of pancreatic juice ever observed in these experiments.

Exp. July 23. R. diaphanes. ♂. Weight 4416g. 1.5 c. cm. Dial injected at 8:15 A.M. Operation from 8:40 A.M. to 9 A.M. Stomach contained a small amount of food. Duodenum cannulated. Tying of the pylorus produced vomiting, also observed in several previous experiments. Pancreatic duct cannulated. The secretion was noted in the divisions of the graduated tube every 30 minutes.

Time.	Secretion in Divisions.
9 A.M. to 9:30 A.M.	0.5
9:30 A.M. to 10 A.M.	4.5
10 A.M. to 10:30 A.M.	2.0
10:30 A.M. 50 c. cm. of digest[1] injected into the duodenum	
10:30 A.M. to 11 A.M.	5.5
11 A.M. to 11:30 A.M.	2.5
11:30 A.M. to 12 noon	8.5
12 noon to 12:30 A.M.	7.5
12:30 P.M. to 1 P.M.	7.0
1 P.M. to 1:30 P.M. / 1:30 P.M. to 2 P.M.	10.0
2 P.M. to 2:30 P.M.	4.0
2:30 P.M. 50 c. cm. of digest injected into the duodenum. The fluid was partly evacuated from the anal opening.	
2:30 P.M. to 3 P.M.	3.5
3 P.M. to 3:30 P.M.	3.5
3:30 P.M. to 4 P.M.	3.0
4 P.M. to 4:30 P.M.	3.0
4:30 P.M. Rectum tied. 50 c. cm. of 0.49 per cent. HCl injected into the duodenum.	
4:30 P.M. to 5 P.M.	3.0
5 P.M. to 5:30 P.M.	12.0
6 P.M. to 6:30 P.M.	13.0
6:30 P.M. to 7 P.M.	11.0
7 P.M. to 7:30 P.M. / 7:30 P.M. to 8 P.M.	16.0
8 P.M. to 8:30 P.M.	10.0
8:30 P.M. to 9 P.M.	7.0

[1] The digest was prepared as follows: Two stomachs of skates and skate's muscles were mixed July 22 with 0.47 per cent. HCl solution and placed in the incubator (10 A.M.). July 23 (9 A.M.) almost all was digested. The fluid acquired a brownish colour. pH of this fluid was 4.2.

Experiment stopped. Animal was in good condition. Amount of pancreatic juice secreted during 12 hours 0.55 c. cm. Its pH = 7.2. Weight of the pancreatic gland 5.35 g. Content of the duodenum alkaline on litmus. Stomach contained water and mucus only, reaction slightly acid on litmus.

The last part of the experiment, *i.e.*, the action of 0.49 per cent. HCl solution, is represented graphically in Fig. 4. The quantities of juice secreted are shown in actual volumes in c.cm. The

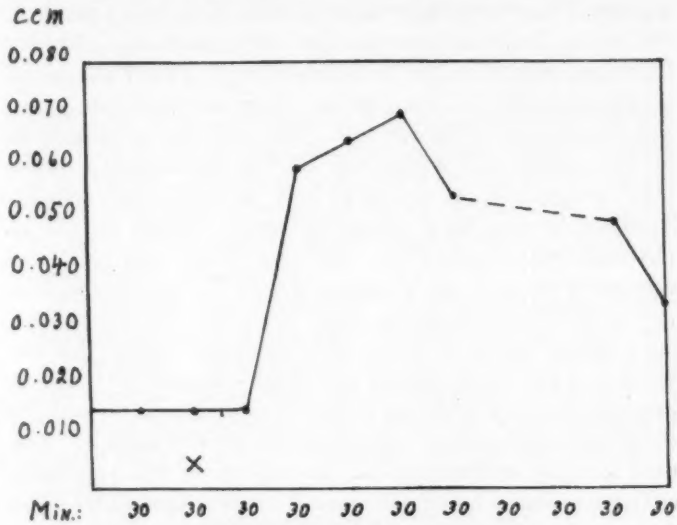

FIG. 4. Curve of the pancreatic secretion in a skate after injection of 0.49 per cent. solution of hydrochloric acid. The ordinates represent the amount of pancreatic juice in c. cm. Every division of abscissa is equal to 30 minutes. At *X* 50 c. cm. of acid was injected into the duodenum.

curve is typical for the action of hydrochloric acid. It is reminiscent of the corresponding curve in warm-blooded animals (dog, man), which generally shows a sharp rise before reaching its peak, and then gradually descends. There is however a stricking difference in the time required for this phenomenon in warm-blooded animals and in the skate. Thus the latent period for the secretory action of HCl in dogs is from 1½ minutes (dogs with a permanent pancreatic fistula) to 4–5 minutes (acute experiments). In skates it requires half-an-hour for the acid to develop its secretory effect. Secretion on 200 c.cm. of 0.5 per cent. HCl in a

dog of 15 to 20 kilos with a permanent pancreatic fistula lasts about an hour and a half to two hours, and somewhat longer in an acute experiment. In a skate of 4 to 5 kilos weight the pancreatic secretion on 50 c.cm. of 0.5 per cent. HCl extends over 4 or more hours. Another feature of the pancreatic secretion in skates is its scantiness as compared with the secretion similarly stimulated in dogs. Thus in the experiment on a skate quoted above the amount of pancreatic juice secreted on 50 c.cm. of 0.5 per cent. HCl during four hours was 0.43 c.cm. In a dog of approximately four times greater weight, a correspondingly greater amount of 0.5 per cent. HCl, *i.e.*, 200 c.cm., in 1 hour, and 45 minutes, gave 138.0 c.cm. of juice (Dolinski (9)). One of the factors responsible for the scantiness of the pancreatic secretion in the skate is its low body temperature as compared with warm-blooded animals. Thus the temperature of the water running through the gils in this experiment was 13.5° C. The water in the tank which was exposed to the air of the laboratory was 15° C., and the temperature of the fish was probably about the same. If Van't Hoff's law could be applied to the secretory processes, namely, that the velocity of a chemical reaction is doubled with each 10° C. rise of temperature, it still could not explain the difference in the activity of the pancreatic gland in warm- and cold-blooded animals. The most probable explanation of the scarcity of the pancreatic secretion in skates is that this organ is not developed to the same degree as in warm-blooded animals. Thus, in dogs, for example, the average weight of the pancreatic gland is approximately $\frac{1}{400}$ of the body weight, whereas in *R. diaphanes* it averages only $\frac{1}{800}$ of the body weight.

No essential difference was noted in the secretory action of the hydrochloric acid alone or when mixed with 2 per cent. urea, one of the permanent constituents of the blood of this fish.

The humoral character of the pancreatic secretion in the skate was emphasized by the secretory action of secretine prepared on 0.36 per cent. hydrochloric acid. The following experiment is quoted as an example:

Exp. July 26. Raja diaphanes. Spinal preparation. Stomach empty. Pan-

creatic duct and common bile duct cannulated. Secretion noted every 15 min. in divisions of graduated tubing.

Time.	Secretion.
3:25 P.M. to 3:40 P.M.	3.5
3:40 P.M. 12:05 c. cm. of secretine injected into one of the gastric veins.	
3:40 P.M. to 3:55 P.M.	2.5
3:55 P.M. to 4:10 P.M.	13.0
4:10 P.M. to 4:25 P.M.	2.0
4:25 P.M. to 4:40 P.M.	3.0
4:40 P.M. to 4:55 P.M.	3
4:55 P.M. to 5:10 P.M.	0

Even after direct introduction into the blood of the secretory agent, *i.e.*, of the secretine, the latent period of secretion was equal to 15 minutes. This fact may probably ve explained by the slowness of the circulation in skates. In the summer time, under the conditions of the experiment, the heart usually contracted only 22 to 26 times in a minute.

The data reported above show that hydrochloric acid in a concentration of about 0.5 per cent. stimulates the pancreatic secretion in skates. The action of the hydrochloric acid is greater after previous introduction into the duodenum of the acid products of protein digestion. The mechanism of the secretory action of HCl is probably humoral, through the formation of secretine.

Experiments with Pilocarpin.

Different kinds of experiments were devised to ascertain the effect produced by pilocarpin on the secretory function of the pancreatic gland, using it as a drug to stimulate the peripheral parts of the parasympathetic nervous system. The following procedures were adopted: (1) Intravenous injection of 0.1 per cent. pilocarpin hydrochloride solution in variable amounts (from 2 to 4 mg. or more). The solution was usually injected with a fine hypodermic needle into one of the gastric veins or into the portal vein itself. (2) Injection of the pilocarpin solution into the conus arteriosus or into the ventricle of the heart. (3) Injection of the solution into the superior mesenteric artery, which supplies with blood the whole dorsal part and half the isthmus of the pancreatic gland, *i.e.* the greater part of the organ. (In sev-

eral experiments the pilocarpin solution was stained with methylene blue.) (4) Introduction of the pilocarpin solution into the pancreatic ducts. For this purpose the graduated tube and the cannula were filled with the solution, and kept in a vertical position for 15 minutes. Under its own pressure the solution entered the duct and distended it. The graduated tube was then returned to the horizontal, and the rate of secretion noted.

In no case did pilocarpin activate the pancreatic secretion or increase spontaneous secretion. Though the negative results in some of the experiments might be explained by the damming back of the pilocarpin solution owing to the narrowness of the small pancreatic ducts, through which the solution could not penetrate to the alveoli, an analogous explanation could not be applied to the experiments where the drug was injected into the arterial system.

As an example of the action of pilocarpin the Experiment of July 30 is quoted.

Exp. July 30. R. diaphanes ♀ Weight 5010 g. 8:15 A.M. Dial injected intraperitoneally. 8:30 to 8:50 A.M. abdomen opened; pancreatic duct cannulated. There was spontaneous pancreatic secretion, which became very insignificant about 1:15 P.M. Skin and muscles covering the heart chamber removed. Pancreatic secretion was noted in divisions of graduated tubing every 15 min. unless marked otherwise.

Time.	Secretion.	Heart Rate Beats per Minute.
1:15 P.M. to 1:30 P.M.	2	
1:30 P.M. to 1:45 P.M.	1	
1:45 P.M. to 2:00 P.M.	0	22
2 P.M. 2 mg. of pilocarpin hydrochloride in 2 c. cm. of distilled water injected into the bulbus aortae. 2.03 P.M. heart 12 beats per minute; 2.06 P.M. heart 18 beats per minute.		
2:00 P.M. to 2:15 P.M.	1	18
2:15 P.M. to 2:30 P.M.	1	18
2:30 P.M. to 2:45 P.M.	1	18
2:45 P.M. to 3:00 P.M.	1	
3 P.M. 2 mg. pilocarpin hydrochloride in 2 c. cm. of distilled water injected into the bulbus aorta. 3.05 P.M. heart 18 beats per minute.		
3:00 P.M. to 3:15 P.M.	0	16
3:15 P.M. to 3:30 P.M.	0	16

3:35 P.M.	4 mg. of pilocarpin hydrochloride in 4 c. cm. of methylene-blue solution injected into the superior mesenteric artery. The main lobe of the pancreatic gland and half the isthmus became blue. Heart rate at 3.40 P.M. 8 per min. Respiratory movements stopped and did not recover till the end of the experiment. Sea water continued to run through the gills. 3:47 P.M. heart rate 14 per min.		
3:35 P.M. to 4:00 P.M.		1	16
4:00 P.M. to 4:15 P.M.		0	8

This experiment shows that pilocarpin introduced into the arterial system in no way stimulates the pancreatic secretion. The heart rate is influenced by pilocarpin, though in far lesser degree than in warm-blooded animals.

Properties of the Pancreatic Secretion.

Although the pancreatic secretion in skates was so scanty (especially in *R. erinacea*), a certain amount of juice (0.2 to 0.5 c.cm.) was obtained in almost every experiment. Pancreatic juice was also collected from the main duct of freshly caught *R. stabuliforis* (in some cases to the amount of 0.5 c.cm.).

The pancreatic juice of the three species investigated is a colourless, almost neutral fluid. The hydrogen ion concentration of the juice determined colorimetrically (Felton's (10) spot method or British Drug Houses Capillator) varied from 6.6 to 7.2 (eleven determinations).

For the determination of its enzymatic activity, the pancreatic juice was usually diluted with distilled water, and the hydrogen ion concentration of the mixture was adjusted to a certain point by means of anhydrous sodium carbonate or corresponding buffer solutions.

These experiments showed that the pancreatic juice of the skate possesses proteolytic, diastatic and lipolytic action. The proteolytic action was increased by adding an extract of the intestinal mucous membrane to the pancreatic juice. The diastatic ferment was effective without any activator. In what form, *i.e.* active or inactive, the pancreatic lipase is secreted one cannot say

since in the method of determination of Anrep, Lush and Palmer (6) sodium glycocholate is used, and this activates the prolipase.

Thus the pancreatic juice of skates contains all three enzymes which are found in the pancreatic juice of higher mammals including man.

To prove conclusively that the pancreatic gland of skates produces these enzymes several experiments were performed with pancreatic extracts. This was the more important since Yung (8) reported that some of the pancreatic extracts of *Scyllium catulus* and *Lamna cornubica* were inactive towards fibrin but always active in the digestion of starch and emulsification of fat. Sullivan (7) could not demonstrate any amylolytic action of water-glycerin extracts of the pancreatic gland of *R. erinacea*.

The pancretic extracts were prepared on 30 per cent ethyl alcohol, the extracts of the intestinal mucous membrane with 0.9 per cent NaCl. They were kept with toluene for several days at room temperature and then filtered through cheese-cloth.

As an example I quote one of the experiments with *R. erinacea*.

Exp. July 22. R. erinacea. The pancreatic gland (weight 1 g.) extracted for three days with 2 c. cm. of 30 per cent. alcohol. July 25, filtered through cheese-cloth. Mucous membrane of the duodenum and of the spiral valve extracted with 0.9 per cent. NaCl for three days.

PANCREATIC AMYLASE.

July 25.	*Control.*
3:50 P.M. 3 drops of pancreatic extract plus 7 drops of 1 per cent soluble starch solution, plus 7 drops of distilled water, plus one drop of toluene, pH = 6.6, in incubator at 37° C.	3:50 P.M. 7 drops of 1 per cent. soluble starch solution plus 7 drops of distilled water, plus one drop of toluene, pH = 6.6, in incubator at 37° C.
7:00 P.M. Reaction with iodine—colorless.	7.00 P.M. Reaction with iodine—blue.
8:00 P.M. Ditto. Fehling distinctly positive.	8:00 P.M. Ditto. Fehling negative.

PANCREATIC LIPASE.

July 26.	*Control.*
11:00 A.M. 3 drops of pancreatic extract plus 2 c. cm. of buffer solution, pH = 8.0, plus 2 c. cm. of glycerol-triacetate, plus 9 drops of sodium glycocholate solution, plus 6 drops of phenol red, plus toluene.	11:00 A.M. Everything in same proportion, except pancreatic extract which was not added.
8:00 P.M. Still pink.	8:00 P.M. Pink.
July 27.	
8:00 A.M. Yellow (pH = 7.0).	8:00 A.M. Pink.

PANCREATIC PROTEASE.

July 27.		*Control.*
10:15 A.M. 5 drops of pancreatic extract, plus 5 drops of distilled water, plus one drop of phenol red, plus one drop of toluene, pH adjusted with Na_2CO_3 to 8.0 and fibrin added.	10:15 A.M. Ditto, plus one drop of intestinal extract.	10:15 A.M. 5 drops of intestinal extract, plus 5 drops of distilled water, plus one drop of toluene, plus fibrin. pH = 8.0.
12:30 P.M. No change.	12:30 P.M. Completely digested.	12:30 P.M. No change.
8:00 P.M. No change.		8:00 P.M. No change.
July 28.		
10:15 A.M. No change. Added one drop of intestinal extract.		10:15 A.M. No change. Added 5 drops of pancreatic extract.
1:00 P.M. Completely digested.		1:30 P.M. Completely digested.

This experiment shows that the pancreatic gland of *R. erinacea* possesses diastatic, lipolytic and proteolytic action. The pancreatic protease is contained in the gland in the form of zymogen.

Experiments with the alcoholic extracts of the pancreatic gland of *R. diaphanes* gave similar results. There were some differences in the rapidity with which the pancreatic enzymes of *R. diaphanes* acted when compared with the action of corresponding enzymes of *R. erinacea*. Thus in the Experiment of August 2, the alcoholic extract of the pancreas of *R. diaphanes* changed the reaction of glycerol-triacetate mixture from pH = 8.0 at 9:10 A.M. to pH = 7.4 at 3:30 P.M., pH = 7.2 at 6:00P.M., and finally to pH = 7.0 at 7:30 P.M. Since the enzymatic strength of extracts prepared from one and the same species of fish varied in different extracts, these variations are to be attributed more to the mode of extracting than to real difference in the content of enzymes in the gland.

One point is worth mentioning. According to Sullivan (7) the water-glycerin extracts of pancreas of different elasmobranch fishes did not digest either the coagulated protein of Mett's tubes or fibrin. These extracts digested gelatine only after activation with water-glycerin extract or chloroform extract of the duodenal mucous membrane, or the extract of the mucous membrane of the spiral valve, the last being the most effective.

As may be seen from this study all pancreatic extracts of *R. erinacea* and *R. diaphanes*, as well as the pancreatic juice of these two species and of *R. stabuliforis*, after activation with the intestinal extract, digested fibrin rapidly.

To verify Sullivan's statement that the mucous membrane of the spiral valve contains more enterokinase than that of the duodenum, special experiments were performed. Samples of the same pancreatic extract *R. diaphanes* were activated with 0.9 per cent NaCl extracts of the mucous membrane of the duodenum and the spiral valve (also *R. diaphanes*). The results were as follows:

The pancreatic extract 15 drops (diluted twice with water and with pH adjusted to 8.0) did not digest fibrin in 48 hours.

The same pancreatic extract in the same dilution with the addition of 4 drops of duodenal mucous membrane extract, digested fibrin in 11 hours.

The same pancreatic extract in the same dilution, plus 4 drops of spiral valve extract, digested the same amount of fibrin in about 20 hours.

Both intestinal extracts alone were inactive towards fibrin.

Thus the duodenal extract showed greater activating power than the spiral valve extract. This was probably due to the presence of a greater amount of mucus in the latter.

Note on the Secretion of Bile

The special arrangement of the experiments in this investigation (tying of the common bile duct and insertion of a cannula into the gall bladder) made it possible to study the bile secretion. The bile (usually dark or emerald green gall-bladder bile in fasting animals) was pressed out from the viscus. The freshly secreted bile was of a straw-yellow color. The reaction of the gall-bladder bile in *R. diaphanes* was slightly acid (average pH = 6.4). The reaction of the gall bladder bile in *R. erinacea* according to Miss Mackay (11) was in average pH 6.3. The reaction of the hepatic bile was slightly alkaline (pH 7.5 to 7.6). The secretion of bile was slow and scanty, although more copious than that of the pancreatic juice. The average rate of bile secretion in fasting *R. diaphanes*, without the application of any stimuli, was from 0.01 to 0.02 c.cm. in thirty minutes. In a successful long experiment more than 1 c.cm. of freshly secreted bile was obtained. In *R. erinacea* the secretion was much slower, producing on the average 0.01 c.cm. per hour.

Introduction into the duodenum of 0.36 per cent. to 0.49 per cent. HCl solutions, in some cases mixed with bile, as well as 10 per cent. Witte's peptone solution, increased the secretion of bile, sometimes doubling it.

Conclusions.

Although the pancreatic gland of the skate secretes the same enzymes as the pancreas of mammals, it seems that in the skate this organ has not attained to the high stage of development of the mammalian pancreas. This is indicated by the scantiness of the pancreatic secretion in skates, the peculiar arrangement of the secondary pancreatic ducts, marked by their narrowness, and the smaller weight of the pancreatic gland in relation to the body weight as compared with warm-blooded animals.

Under the experimental conditions described the pancreatic secretion in skates is continuous but scanty. The hydrogen ion concentration of the pancreatic juice is equal to pH 6.6 to 7.2. This again is a special feature of the secretion, since the pancreatic juice in mammals (dog, cat, man) is decidely alkaline (average pH = 8.4). Whereas in mammals the pancreatic juice plays an important part in the neutralisation of acid chyme entering the duodenum, in skates the scanty and almost neutral pancreatic secretion cannot be an important factor in this respect. The reaction of the gall-bladder bile in skates is slightly on the acid side, and that of the hepatic bile very faintly alkaline. Nevertheless the reaction in the duodenum is strongly alkaline, this being evidently due to the alkaline secretion of the succus entericus

Hydrochloric acid introduced into the duodenum activates the pancreatic secretion, probably in a humoral way, since intravenous injections of secretine produce a positive secretory effect. Parasympathetic poison, such as pilocarpin, does not influence the pancreatic secretion in any way.

Summary.

1. The system of pancreatic ducts in *R. erinacea*, *diaphanes* and *stabuliforis* presents certain peculiarities which differentiate it from the analogous system of ducts in higher mammalian animals.

2. Pancreatic secretion in skates which have previously fasted

for several days is continuous but very scanty. Hydrochloric acid and secretin increased this secretion. Pilocarpin was without effect. The previous introduction into the duodenum of the acid digest of proteins increased the secretory effect of the hydrochloric acid.

3. The pancreatic juice is a neutral fluid (pH = 6.6 to 7.2), possessing proteolytic, diastatic and lipolytic action.

4. Alcoholic extracts of the pancreas show the same enzyme action as the juice. A proteolytic enzyme is contained in the gland in the form of protrypsin, which may be activated by 0.9 per cent. NaCl extract of the mucous membrane of the duodenum and spiral valve.

5. Bile is secreted continuously. Introduction of 0.36 per cent. HCl solution and 10 per cent. Witte's peptone solution into the duodenum increases the secretion.

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THE RESISTANCE OF THE FRESHWATER SNAIL, *PHYSA HETEROSTROPHA* (SAY) TO SEA WATER.

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The freshwater snail *Physa heterostropha* has been observed in the brackish water of Barnegat Bay near the mouth of the Metedeconk River, Bay Head, N. J. An interesting problem presented itself; how far into brackish water can these freshwater snails migrate? To test this some preliminary experiments were attempted in the summer of 1928. By gradually increasing the salinity of the water *Lymnæa stagnalis appressa* and *L. palustris* (from Michigan) were made to live in 25 per cent. sea water. *Physa heterostropha* (from Philadelphia) died soon after being placed in 10 per cent. sea water. This stock was probably weak. These experiments were summed up in a preliminary report (Richards, 1929).

The summer experiments were of a very preliminary nature and rather crude; so additional and more accurate experiments were begun in the fall of the same year. The work, which was concentrated upon the one species, *P. heterostropha*, seemed to divide itself into two parts: first the experiments dealing with the ability of the species as a whole to withstand gradual and sudden increases in the concentration of sea water; and second, the reactions of the various stocks of the same species to the salt water.

The writer wishes to express his thanks to Dr. H. Burrington Baker and Dr. Edward D. Crabb, both of the University of Pennsylvania, for suggestions and valuable information as to the proper food for the snails and for help in other ways.

METHODS AND PROCEDURE.

In order to determine whether there were any racial differences between various lots of the same species, several sets of controls were kept.

Control.	Locality.	Date collected.
A	Schuylkill River, Fairmount Park, Philadelphia, Pa.	Oct. 16, 1928
B	Schuylkill River, Fairmount Park, Philadelphia, Pa.	Oct. 27, 1928
C	Branch of Cobbs Creek, Haverford, Pa.	Oct. 28, 1928
D	Davies Lake, Cape May Point, N. J.	Nov. 11, 1928
E	Davies Lake, Cape May Point, N. J.	Mar. 30, 1929
F	Wissahickon Creek, Fort Washington, Pa.	Apr. 6, 1929
G	Pond, Botanical Gardens, University of Pennsylvania, Philadelphia, Pa.	Aug. 7, 1929
H	Davies Lake, Cape May Point, N. J.	Aug. 11, 1929

The snails were brought to the Zoölogical Laboratory of the University of Pennsylvania, where they were placed in culture jars. The water used in these experiments was tap water which had been allowed to stand for several days in order to eliminate some of the chlorine so abundant in Philadelphia tap water. In all cases the snails were allowed to live in the fresh water for one week in order to adjust themselves to any possible change. The mortality of the control was found to be highest during the first week.

After the first week some snails were placed in 5 per cent sea water,[1] and a 5 per cent. increase per week was continued until the water reached 30 per cent.; at this point serious effects of the salts were noticed in the behavior of the snails and therefore some were allowed to remain in this concentration with the hope that they might become adjusted to it; others were placed in stronger sea water.

Additional experiments were attempted in which the 5 per cent. increase was made at intervals of two days and one day. Other snails were placed directly in 5 per cent., 10 per cent., 15 per cent., 20 per cent. and 25 per cent. sea water in order to see if they could stand the sudden change.

The sea water used to make up these solutions in all the experiments except part 3 was taken from the vivarium of the University of Pennsylvania. This water had been brought from Ocean City, N. J., some months previous. In the experiments of part 3, which were conducted at Cape May Point, N. J., water from the ocean was used.

The specific gravity of the vivarium water is kept relatively constant at 1.023 (at 17.5°C.). The principal difference between

[1] Normal sea water being taken as 100 per cent.

vivarium water and normal sea water was found to be in the hydrogen ion concentration. Normal sea water (off New Jersey) has a pH between 8.1 and 8.3, whereas the water in the tank is more acid, varying from pH 7.8 to 7.9. The acidity is probably due to the acid excrement and dead organic matter from the marine animals in the aquarium.

The snails were fed green lettuce several times a week, and the old lettuce and excrement removed at frequent intervals, the procedure recommended by Crabb (1929) for the best growth of pond snails.

The temperature was not regulated; it usually lay within the interval between 15° and 20° C.

Results.

Part 1, *5 per cent. Increase in Concentration Every Week.*

Physa lived actively in water as strong as 25 per cent. sea water. Above 25 per cent. the harmful effects of the salts were noted. Above this concentration the activity of the snails was considerably decreased. Snails from control *D* (Cape May Point, N. J.) were considerably more active in 30 per cent. sea water than those from any other control.

Snails were allowed to remain in water of between 25 per cent. and 30 per cent. for several weeks in order to see if they gradually became adjusted to this concentration. However no such adjustment seemed to take place during the several weeks, although throughout the experiments the snails from control *D* were more active in the brackish water than any of the others.

After various intervals the snails were placed in higher concentrations (up to 50 per cent.). In all cases they died, except those in *D*, which were inactive but still alive. Upon being transferred to 5 per cent. sea water they soon revived and were as active as ever.

These experiments are summarized in the following two tables. Table I. deals with the experiments with the 5 per cent. increase in concentration as far as 30 per cent., at which point this method was discontinued.

TABLE I.

SHOWING RESULTS OF EXPERIMENTS IN WHICH THE 5 PER CENT. INCREASE IN CONCENTRATION WAS MADE EVERY WEEK.

The number of snails alive at the beginning and end of each week is given. The summation of all experiments is also given.[1]

		5%	10%	15%	20%	25%	30%
A control	33–19	13–11	11–11	11–11	11–11	11–11	11–11
Experiment		5–5	5–5	5–5	5–5	5–5	5–5
B control	70–67	52–52	52–47	47–45	45–45	40–40	40–40
Experiment		10–10	10–10	10–10	10–10	10–10	10–10
C control	149–139	100–95	95–93	83–83	83–83	83–83	83–83
Experiment		15–15	15–15	15–15	15–15	15–15	15–15
D control	50–50	50–50	50–50	50–50	50–50	50–50	50–50
Experiment		10–10	10–10	10–9	9–9	9–9	9–9
Summation controls	302–275	215–208	208–201	191–189	189–189	184–184	184–184
Experiments		40–40	40–40	40–39	39–39	39–39	39–39
% surviving controls	91.0%	92.3%	96.6%	98.9%	100%	100%	100%
Experiments		100%	100%	97.5%	100%	100%	100%

Table II. surveys the experiments in water of higher concentration than 30 per cent. Since the procedure following the period of attempted adjustment differed in the various experiments, and would be difficult to represent in strictly tabular form, the procedure and results are summarized in a few words in Table II.

Part 2, *5 Per Cent. Increase in Concentration at Intervals of Two Days.*

When the concentration was increased 5 per cent. every two days instead of every week, the results were practically the same. In these experiments also the snails from Davies Lake (*D* and *E*) showed a greater resistance to sea water than the other snails.

The results of these experiments are shown briefly in Table III.

[1] For the sake of comparison the following table is given showing the average specific gravity (at 17.5° C.) and average salinity (parts per thousand) of the various concentrations of sea water used throughout these experiments. The data were calculated from Knudsen's Hydrographical Tables.

Per Cent. Normal Sea Water.	Sp. Gr. (17.5° C.).	Salinity.	Per Cent. Normal Sea Water.	Sp. Gr. (17.5° C.).	Salinity.
5%	1.0007	1	30%	1.0085	12
10%	1.0020	2 to 3	35%	1.0105	14.5
15%	1.0040	4 to 5	50%	1.0130	17
20%	1.0050	6	100%	1.0230	30
25%	1.0070	8			

TABLE II.

SHOWING EXPERIMENTS IN WATER OF HIGHER CONCENTRATION THAN 30 PER CENT.

The number of snails alive at the end of the week in 30% is given, then the number of weeks that the snails were left in water of approximately 25 or 30%; the further experiments are then summarized in a few words.

	Number Alive in 30% (from Table 1).	Number of Weeks in 25–30%.	Further Experiments.
A control.....	11		11–11
Experiment 1...	5	1	Placed in 40%; death at end of one week.
B control.....	40		40–38
Experiment 1...	5	4	Placed in 35%; one dead at end of first week; others died during second week.
Experiment 2...	5	1	Increased to 35% in which three died; increased to 50%; all died.
C control.....	83		83–83
Experiment 1...	5	2	Concentration fluctuated considerably between 1.007 and 1.012; died in four weeks.
Experiment 2...	10	2	Active in 30%; alive but inactive in 35%; died in 50%.
D control.....	50		50–50
Experiments....	9	3	Active in 30% and 35%; placed in 50% alive but inactive at end of 24 hours; revived when placed in 5% sea water.

TABLE III.

SHOWING RESULTS OF EXPERIMENTS WITH INCREASE OF 5 PER CENT. IN SALINITY AT INTERVALS OF TWO DAYS.

The number of snails alive at the beginning and end of each interval is shown

	5%	10%	15%	20%	25%	30%	35%	40%	50%
B Control...	8–8	8–8	8–8	8–8	8–8	8–8	8–8	8–8	8–8
Experiment..	5–5	5–5	5–5	5–5	5–5	5–5 fairly active	5–5 inactive	5–2 inactive	2–0 dead
C Control...	8–8	8–8	8–8	8–8	8–8	8–8	8–8	8–8	8–8
Experiment..	5–5	5–5	5–5	5–5	5–5	5–5 fairly active	5–5 inactive	5–5	5–0 dead
D Control...	21–21	21–21	21–21	21–21	21–21	21–21	18–18	18–18	18–18
Experiment..	10–10	10–10	10–10	10–10	10–9	9–9 active	9–9	9–9 fairly active	9–8 slightly active. Revived in fresh water.
E Control...	16–16	16–16	16–16	16–16	16–16	16–16	16–16	16–16	16–16
Experiment..	5–5	5–5	5–5	5–5	5–5	5–5	5–5	5–5 active	5–5 fairly active. Revived in fresh water.

Part 3, *5 Per Cent. Increase in Concentration at Intervals of One Day.*

There was no difference in the behavior of the snails when the increase was made at intervals of one day instead of two days. Those snails (*H*) from Davies Lake showed slightly greater resistance to sea water than those (*G*) from the pond in Philadelphia.

Part 4, *Sudden Change to Brackish Water.*

Physa was able to live after sudden transfers to 5 per cent., 10 per cent., 15 per cent., 20 per cent. and 25 per cent. sea water, but the Davies Lake snails (*D*, *E* and *H*) were the only ones to show signs of activity in 20 per cent. and 25 per cent. sea water.

Discussion.

Whether the concentration was increased 5 per cent. at intervals of one or two days or even a week did not seem to have any significant bearing on the ability of the snails to become acclimated to the sea water. Various workers have achieved complete acclimatization of freshwater organisms to sea water over a long period of time. Beaudant (1816) successfully acclimatized *Physa fontinalis* to sea water by very gradually increasing the salinity of the water over a period of more than six months. A very long period of time, and a very gradual increase in the concentration of the salt are probably necessary for the complete acclimatization of most freshwater organisms.

Hydrogen Ion Concentration.

The possibility that death might have been caused by changes in pH, rather than by increase in salinity, was considered. Walton and Wright (1926) have shown that *Lymnæa truncatula* can stand a variation in H-ion concentration from pH 6.0 to pH 8.6, and *L. peregra* from pH 5.8 to pH 8.8. All the solutions used in the experiments lie well within this range as the following table will show:

	pH
Tap water	7.2–7.4
Aquarium water	7.2–7.4
Control after one week	7.2–7.3
Sea water from Vivarium	7.8–7.9
Sea water (Cape May Point, N. J.)	8.1–8.3
25 per cent. sea water (diluted with aquarium water)	7.5–7.7

The pH was determined colorimetrically by the use of the indicators Cresol Red and Brom Thymol Blue, and was verified in a few cases electrolytically. *Physa* was able to live normally when transferred to the acid Cedar Swamp water from the Mullica River near Batsto, N. J. (pH 5.9); likewise the snails could live in synthetic alkaline water (pH 8.1).

These data serve to show that probably some effect of the salts present in sea water rather than the change in H-ion concentration causes the death of the snails.

A More Hardy Stock from Davies Lake.

Numerous indications throughout the experiments showed that the snails from Davies Lake, Cape May Point, N. J. (controls *D*, *E* and *H*) have more resistance than the snails from the other localities. They were more active than the others in 25–30 per cent. sea water; they were active in water as concentrated as 1.012 (at 17.5 °C.), showing a resistance not shared by the other stocks; they were the only stock to survive 50 per cent. sea water; here they were inactive, but soon revived when placed in 5 per cent. sea water; they were the only stock to show any signs of activity after a sudden transfer from fresh water to 20 per cent. and 25 per cent. sea water.

This evidence seems to show fairly well that this stock is more resistant to sea water than those taken from the vicinity of Philadelphia. Although several tests of water from Davies Lake showed a specific gravity of 1.000 at 4° C., during storms the waters of the lake are probably mixed with the salt water of Delaware Bay only 50 yards distant. The resistance of the Davies Lake stock may have been acquired during several generations.

Bailey (1929) has recorded *P. heterostropha* together with other freshwater snails in the brackish water of Chesapeake Bay, and as mentioned above, the writer has found the same species in Barnegat Bay, N. J.

SUMMARY.

1. By gradually increasing the salinity, *Physa heterostropha* was made to live quite actively in 25 per cent. sea water.

2. *Physa* can live but is not active in concentrations between 30 per cent. and 40 per cent.

3. In water stronger than 40 per cent. all the snails died with the exception of the Davies Lake lot, which had retreated within their shells, but which revived when placed in 5 per cent. sea water.

4. Whether the 5 per cent. increase in concentration was made at intervals of one or two days, or even a week, seemed to make no difference in the ability of the snails to become acclimated to the sea water.

5. All the races survived a sudden change to 5 per cent., 10 per cent., 15 per cent., 20 per cent. and 25 per cent., but the Davies Lake stock was the only one active in 20 per cent. and 25 per cent., at the end of one week.

6. The snails from Davies Lake may be regarded as a stock which is more hardy to sea water than the other stocks used in the experiments.

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CHANGES IN pH OF ALBUMEN AND YOLK IN THE COURSE OF EMBRYONIC DEVELOPMENT UNDER NATURAL AND ARTIFICIAL INCUBATION.

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Considering the influence of acidity on colloidal properties of organic substances, such as animal fluids, tissues, etc., it is important to recognize the significance of pH value in various parts of the developing bird's egg. The most essential parts of the egg are albumen and yolk; they furnish almost all the food material to the growing embryo during the entire period of incubation. They must have a proper pH value. There is evidence to warrant the belief that any variation in pH of albumen and yolk from a normal value, particularly when affected by an environment, such as carbon dioxide gas, will develop a pathological condition within an egg and in extreme cases, will cause the death of the embryo. Therefore, it is important to establish the normal curves of pH value for both egg constituents in order to assist in further comparative studies of the growth and metabolism of the embryo under various environmental conditions of artificial incubation.

The literature relating to the subject shows that Aggazzotti, ('13), presumably, was the first who observed pH value of albumen and yolk in incubated eggs. Then a few observations were made by Gueylard ('25), Healy and Peter ('25), and, on egg albumen alone, by Wladimiroff ('26). Unfortunately, the data of the above investigators, being either insufficient or inconsistent, do not give a complete picture of all changes in pH value of the egg constituents during the incubation period.

The present paper concerns itself with a study of pH value of albumen and yolk in the developing egg throughout the incubation period under both natural and artificial (experimental) incubation.

Methods and Materials.

The eggs used were from White Leghorn hens. They were selected for uniformity of size, shape, and shell texture, and incubated soon after laying (in spring, 1929). In one experiment the eggs were incubated by the natural method, under sitting hens; in another experiment, by an artificial method, in a special electric laboratory incubator (Romanoff, '29). All physical factors of artificial incubation were predetermined and kept constant. The temperature was 38.0 ± 0.2° C.; the relative humidity 62.5 ± 1.0 per cent; the inside ventilation was 0.5 cu. ft., and fresh air from outside was added to give an adequate supply of oxygen. The carbon dioxide content was increasing from 0.15 to 0.65 per cent. The eggs were turned three times a day.

In the experiments, at intervals of twenty-four hours, usually four eggs with normally-developed embryos were removed for analysis. The pH values of egg albumen and yolk were determined electrometrically, using hydrogen electrode. The observation of yolk was carried on up to the hatching time, and that of albumen up to the sixteenth day of incubation, as long as it was available. After that time the egg albumen normally enters the yolk sac and loses its physical appearance.

Results and Discussion.

In a study of fresh eggs (just laid, warm, and without an air cell) it was found that the pH value of albumen and yolk was on an average 7.827 ± 0.046 and 5.973 ± 0.015. A slight difference was noticed in pH value of the outer and middle layers of egg albumen, examined separately. This is illustrated by the data of a few analyses (Table I.).

Table I.

Hydrogen-ion Concentration in Fresh Eggs.

Egg Number.	Layers of Egg Albumen.		Egg Yolk.
	Outer.	Middle.	
	pH	pH	pH
1	7.929	7.890	5.960
2	7.995	7.951	5.977
3	8.001	7.946	5.968
Average	7.975	7.929	5.968

The middle layer, being close to the yolk (Fig. 1), was invariably less alkaline than the outer one. In incubation as soon as an egg is heated the different layers of albumen disappear, and the separ-

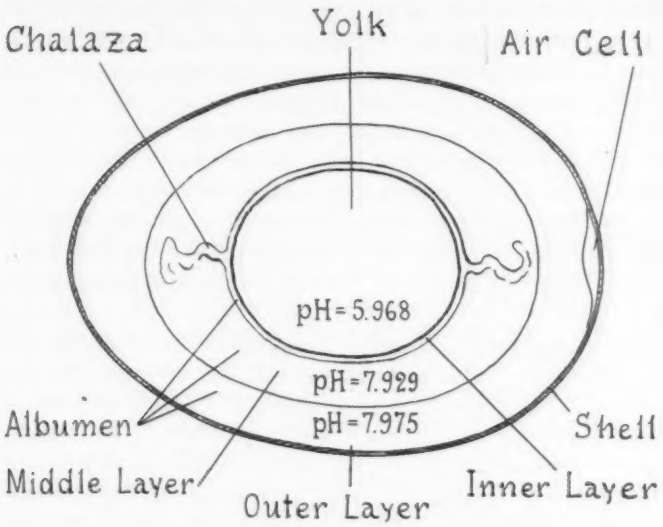

FIG. 1. Hydrogen-ion concentration of a fresh egg.

ate analyses can not be performed. No attempt was made to determine pH value of the inner layer, because of its extremely small size.

The data of our experiments on pH value of egg albumen under natural and artificial incubations, together with the data of previous workers, are shown in Table II.

It is evident, particularly from our data (Fig. 2), that egg albumen rapidly becomes alkaline, reaching the highest point at about two days of incubation. Then it turns towards neutrality, reaching the original pH value at about six days of incubation. A slight difference in pH value at two days, under natural and artificial methods, may be explained primarily by the influence of environmental conditions of incubation. Presumably, the carbon dioxide concentration was lower in the laboratory incubator than under the sitting hen. This difference can not exceed 0.1 per cent. of carbon dioxide concentration.[1]

[1] A paper on "Effect of Carbon Dioxide on pH of Albumen in the Developing Egg," has been prepared for publication. (Will appear in *Jour. Exp. Zool.*)

The data of our experiments on pH value of egg yolk under both methods of incubation, with the data of other workers, are shown in Table III.

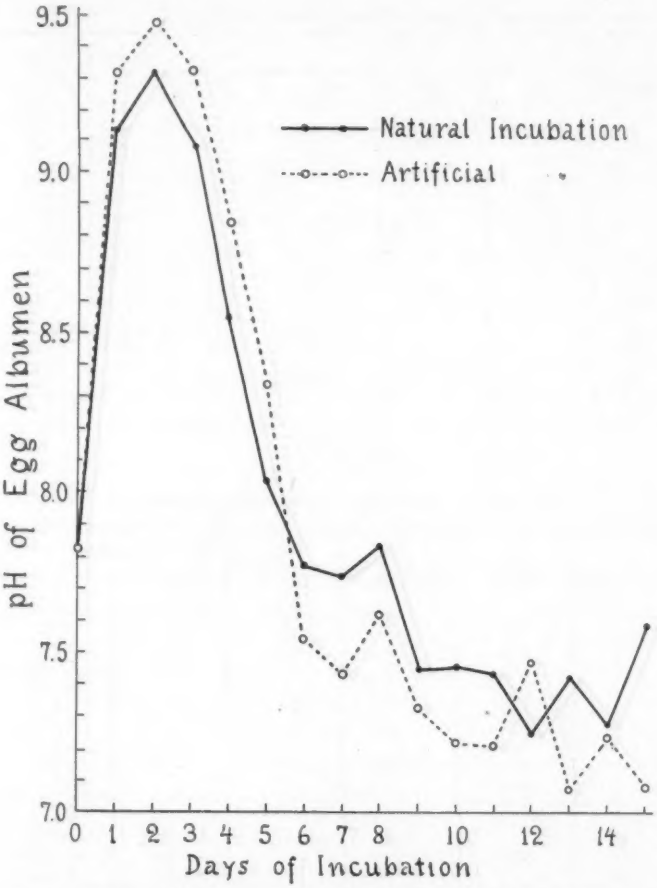

FIG. 2. Changes in pH of egg albumen under natural and artificial (experimental) incubation.

The pH value found by Aggazzotti ('13) seems to be consistently lower than ours. His data, being incomplete, do not demonstrate the pronounced drop at 16 days of incubation. It is seen

TABLE II.

CHANGES IN pH OF EGG ALBUMEN DURING INCUBATION.

Incub. Age.	Aggazzotti (1913).	Gueylard & Portier (1925).	Healy & Peter (1925).	Wladimiroff (1926).	Authors.	
					Art. Incub.	Nat. Incub.
(days)	pH	pH	pH	pH	pH	pH
0	8.66	7.97	8.24		7.827	7.827
1	8.34				9.317	9.157
2	8.38	8.60		9.36	9.472	9.311
3	7.91		9.4	9.46	9.318	9.080
4	7.40			9.19	8.835	8.546
5	7.82			8.72	8.343	8.038
6	7.41		8.2	8.66	7.536	7.785
7	7.71			8.57	7.414	7.748
8	7.24			7.88	7.614	7.840
9	7.34			8.29	7.328	7.450
10		7.25		7.82	7.255	7.466
11	6.61			7.88	7.231	7.435
12				7.55	7.473	7.257
13				7.47	7.079	7.419
14	6.14				7.247	7.283
15				7.57	7.086	7.593
16				7.61		

TABLE III.

CHANGES IN pH OF EGG YOLK DURING INCUBATION.

Incub. Age.	Aggazzotti (1913).	Gueylard & Portier (1925).	Healy & Peter (1925).	Authors.	
				Art. Incub.	Nat. Incub.
(days)	pH	pH	pH	pH	pH
0	4.06	5.48	6.36	5.973	5.973
1	4.83			6.087	6.034
2	4.04	5.73		6.151	6.082
3	4.37		6.8	6.215	6.234
4	5.55			6.286	6.117
5	5.73			6.445	6.541
6	6.12	7.54	6.8	6.631	6.589
7	5.99			6.667	6.872
8	5.96			6.756	6.946
9	5.85			6.750	7.007
10		6.83		6.855	7.327
11	6.68			6.926	7.430
12				7.050	7.450
13	6.82	7.42		7.043	7.772
14	6.98	7.08		7.042	7.920
15				6.899	8.099
16				6.105	7.401
17	6.83	4.12		7.693	7.820
18	6.26	5.64		7.701	7.637
19	6.64			7.714	7.608

from the curves of pH value under natural and artificial incubation (Fig. 3) that egg yolk, from its original acid state, gradually goes to a neutral, and then to a slightly alkaline state. The

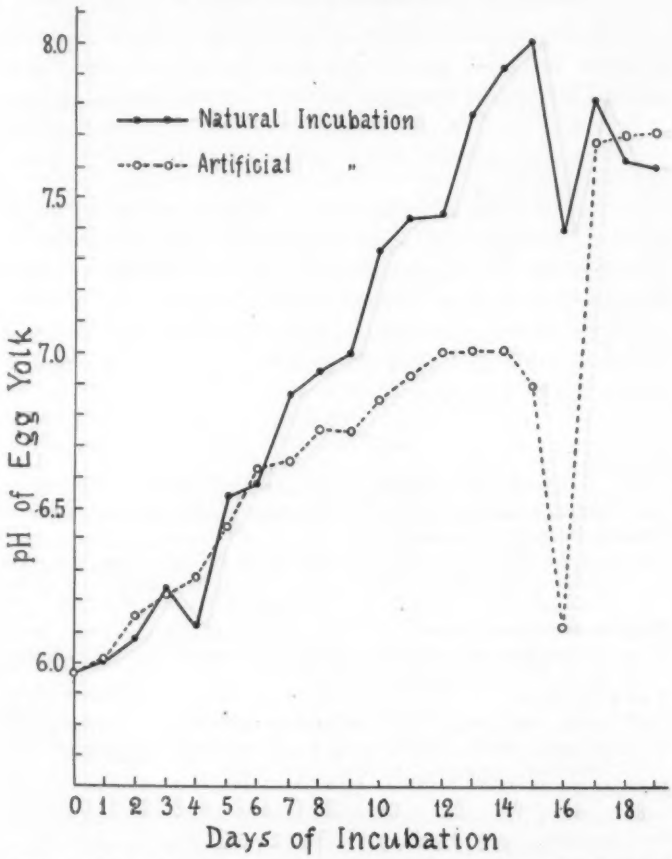

FIG. 3. Changes in pH of egg yolk under natural and artificial (experimental) incubation.

above-mentioned sudden drop in pH value under both natural and artificial incubation is possibly related to the natural depression of the growth in the life span of the embryo (Romanoff, '29*a*). But the difference in pH value under natural and artificial meth-

ods during the period from 7 to 16 days of incubation may be explained, as suggested above by the difference in carbon dioxide concentration.

SUMMARY.

1. In the course of embryonic development the pH values of albumen and yolk go through definite changes, presumably affected by natural metabolic processes occuring within an egg.

2. It was found that the changes in pH either of albumen or of yolk were similar under natural and artificial (laboratory) methods of incubation.

3. The pH of egg albumen rapidly changed towards alkalinity and back during the first week of incubation, reaching the highest point of alkalinity at about 48 hours. For the rest of the incubation period it gradually moved towards neutrality.

4. The pH of egg yolk gradually changed throughout the incubation period from acid to alkaline, with a sudden temporary drop at the sixteenth day.

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BREEDING HABITS OF *NEREIS DUMERILII* AT NAPLES.[1]

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In his monograph on the natural history of *Nereis dumerilii*, Hempelmann has given us a very complete account of the life cycle of this annelid. My own work on the life history of *Platynereis* was stimulated by his classic study. During some years of experience in rearing sexually mature *Platynereis* from inseminated eggs normally laid, I was never able, under laboratory conditions, to establish for this form a life history comparable to that of *N. dumerilii*. I drew my conclusions with some reservation, stating that it might still be possible in nature that *Platynereis* goes through such a life cycle. When in addition I later made some experiments which indicated the possibility of sex reversal in sexually immature *Platynereis*, I became more sceptical of the earlier work: it might be that what appeared as sex-reversal was merely the exaggeration of the female condition, in normally hermaphroditic individuals, to the extinction of the male sex. If there were normally hermaphroditic *Platynereis* which I had previously overlooked, it was possible that I had also failed to observe other stages in the life cycle comparable to those described by Hempelmann. Finally, when Prof. J. Percy Moore suggested to me that *Platynereis megalops* and *Nereis dumerilii* might be identical, I was indeed ready to discard all of my results on *P. megalops*.

One approach to the problem seemed evident: namely, to study the breeding habits and development of *N. dumerilii* at Naples where Hempelmann had conducted his researches, although I had implicit confidence in the validity of his findings. I may say at once that so far as my studies went, they completely confirm Hempelmann's. It would have been nothing short of

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amazing if, for example, this painstaking worker had overlooked such an obvious and striking phenomenon as the breeding behavior exhibited by *Platynereis megalops*. After almost ten years of waiting I was able in 1929 to spend several months at the Naples Zoölogical Station with the primary object of making observations on *N. dumerilii*. Their results are here reported.

It is a very great pleasure to take this opportunity to thank Prof. R. Dohrn, Director of the Naples Zoölogical Station, for his many kindnesses which made my stay most profitable. I also wish to acknowledge my great indebtedness to Prof. Dr. Josef Spek for placing at my disposal valuable information, the result of his own experience, concerning the care of *N. dumerilii* in the nereid phase.

The Observations.

The observations on breeding habits of *Nereis dumerilii* at the Naples Zoölogical Station during the period January to June, 1929, fall into two categories: first, observations on animals reared to sexual maturity in the laboratory; and second, those made on sexually mature animals during the swarming period.

Sexually immature specimens of *N. dumerilii* brought to the laboratory were kept in dishes of sea-water with diatoms and algæ in which the worms formed tubes. They were examined daily and isolated when found, by the appearance of the parapodia, to be nearly sexually mature. At maturity the animals were observed in pairs, the sperm and egg shedding noted.

Repeated observations of this kind made during February and March revealed that the sperm-shedding and egg-shedding processes are almost identical with those described by Lillie and Just for *Nereis limbata* at Woods Hole, Mass. Briefly, the presence of a mature female or its eggs in sea-water induces sperm shedding by the male when placed in the dish containing the female or its eggs. In three cases males shed sperm when placed in a dish with a female that externally appeared sexually mature but whose eggs neither in structure nor in fertilization capacity were physiologically "ripe"—that is, on addition of a drop of active sperm from suspensions, known by trial inseminations on eggs from other females to be in optimum condition since fertilization resulted—no egg developed. This was never found to be the case with *Nereis*.

Also, the presence of sperm in sea-water induces oviposition. Whenever ripe females were placed in a suspension of active spermatozoa, oviposition invariably followed. When this did not take place the eggs were in poor condition: they failed to show any signs of development following insemination with active sperm.

The sperm and egg shedding process of *N. dumerilii* thus closely resemble if they are not identical with those of *N. limbata*. It is interesting also to note that the eggs of the two forms show a close resemblance: in form, distribution and number of oil drops, and in pigmentation. I did not measure the eggs, but Professor Spek informed me that they are 100 μ in diameter. Eggs of *N. limbata* are about 87.5 to 100 μ. On the other hand, these sexually mature worms are morphologically different from *N. limbata*. Indeed they more closely resemble *Platynereis megalops*.

I have never seen a swarming of *N. limbata* to equal that of *N. dumerilii* at Naples after the full moon of May twenty-third. Within the circle of the light, the sea seemed alive with worms. One evening at Capri and also once at Ischia I also observed the swarming of *N. dumerilli*. During the swarming period the animals exhibit precisely the same breeding behavior described for *N. limbata*. Around the slowly swimming females, the active males move with ever increasing rapidity and more closely set spirals. Frequently, several males would thus swim over and under a female. They then discharge sperm. Into the cloud of sperm the female discharges her eggs and sinks to the bottom of the sea. In the laboratory, males and females—separated at the time of capture—when placed together, went through the same performance. *There was never any indication of the copulation so characteristic of Platynereis megalops.*

This note on breeding habits of *Nereis dumerilii* at Naples is sufficient to show that this annelid behaves almost identically as *N. limbata*. If *N. dumerilii* and *Platynereis megalops* are the same, either Hempelmann in his careful observations, made during a prolonged stay at Naples, missed that form in the life cycle of *N. dumerilii* which lays eggs only after copulation, as does *Platynereis megalops;* or, differences in the American environment are responsible for the peculiar breeding habits of *Platynereis megalops*. I am loathe to accept the first alternative; the second

I consider unlikely. It might be argued that my own work is far too incomplete to warrant any assumption that I have definitely settled this question. With this I would heartily agree. However, it is permissible, I think, to conclude that as far as they go, my observations indicate that *Nereis dumerilii* and *Platynereis megalops* are not the same species.

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THE PRODUCTION OF FILAMENTS BY ECHINODERM OVA AS A RESPONSE TO INSEMINATION, WITH SPECIAL REFERENCE TO THE PHENOMENON AS EXHIBITED BY OVA OF THE GENUS *ASTERIAS*.[1]

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In 1923 Chambers recorded results which constituted "an attempt to explain the peculiar behavior of the starfish spermatozoön which enables it to migrate through the jelly of the egg." On the basis of my observations extending over a period of years on living and sectioned eggs of the genus *Asterias*, I was forced to disagree somewhat with Chambers' results. The main point of this disagreement concerned itself with Chambers' time-table of the events in the process of sperm entry which follow insemination of the egg. His observations I may briefly summarize as follows:

Twenty-seven minutes after insemination the spermatozoön at the outer boundary of the egg jelly is in contact with a fine filament which grows out from a conical elevation on the surface of the egg through the intervening jelly. The spermatozoön approaches nearer the egg surface and reaches the cone; one gains the impression that the cone exerts a pull on the spermatozoön (two minutes after insemination.) With the arrival of the spermatozoön at the summit of the cone, there ensues a pause of about thirty seconds. The sperm-head narrows at its tip, lengthens, and rapidly slips through the vitelline ("fertilization") membrane which, one minute after insemination, had separated from the egg surface (two minutes and thirty-three seconds after insemination). Within the substance of the cone the sperm-head rounds up (two minutes and thirty-four seconds after insemination).

On the other hand, I have repeatedly observed in both the living and sectioned eggs (killed during the period of three minutes

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after insemination at five second intervals) that: (1), spermatozoa have reached the egg surface through the jelly *five seconds* after insemination; (2), spermatozoa are well attached to the vitelline membrane *fifteen seconds* after insemination; (3), the vitelline membrane begins to separate from the egg surface *forty-five seconds* after insemination at which time the entrance cone is well defined within the perivitelline space; and (4), the spermatozoön enters the cone about *two minutes* after insemination. With respect to sequence of events in the process of sperm entry, my data are thus in serious conflict with those of Chambers. The question was therefore raised whether the manipulations of the ova inherent in his methods of examination might not have produced abnormal behavior. (See Lillie and Just, 1924, pp. 458–559.)

In my judgment, however, there is an equally serious question, quite apart from that of the micro-dissection method, concerning this work of Chambers on the mechanism of the entrance of the spermatozoön into the starfish egg. Though the main interest throughout my studies of fertilization in the genus *Asterias* has been the *normal* process of sperm entry in ova in optimum fertilizable condition, I have from time to time noted the production of filaments in ova of this and other echinoderms as a response to insemination. Such productions, I believe, are the response of abnormal ova. The present communication aims to set forth the evidence on which I base this belief.

Observations.

The formation of filaments (or papillæ) as a response to insemination have been observed in ova of the following echinoderms: *Thyone briareus*, *Strongylocentrotus dröbachiensis*, *Arbacia punctulata*, *Asterias forbesii*, and *A. vulgaris*. The observations were made during several spring and summer months at the Marine Biological Laboratory, Woods Hole, Mass. More recently, during the winter months of 1929, while enjoying the privilege of working at the Zoölogical Station of Naples, I also made observations on the ova of *Asterias glacialis*.

We may begin the description of the production of filaments by ova as a response to insemination with an account of the phenomenon in the ovocytes of *Thyone briareus*.

Eggs of *Thyone* are readily inseminated if they have been naturally shed by the animals. The animals, easily obtainable in abundance, spawn actively throughout the breeding season, provided they are kept in large quantities of sea-water. Pearse ('09) has recorded an observation on the spawning of *Thyone*. These normally shed eggs, as observations made repeatedly during the month of June of several years revealed, never form protoplasmic filaments as a response to insemination. During April and May, 1927, however, I was always able to observe the extrusion of filaments from the cortex of *ovocytes* on insemination. In most cases the surface of the ovocytes literally bristled with the delicate filaments extending beyond the striated egg-jelly. But these ovocytes never developed.

The formation of protoplasmic filaments by holothurian ovocytes in the presence of spermatozoa has been previously reported by Hobson who studied the phenomenon in *Holothuria nigra*.

Strongylocentrotus dröbachiensis. During the spring months of 1927 and 1928 I observed the formation of filaments by the mature eggs of this echinid on the addition of spermatozoa to the water containing them. These were the most beautiful and numerous filaments that I have ever seen. However, in 1927, of the eggs from thirty-three females, less than 0.7 per cent. cleaved; and no egg went beyond cleavage. The experience of 1928 was the same. The explanation is simple: the animals came to me from cold sea-water, forty-eight hours after collection, closely packed in wet sea-weed. When the inseminations were made in sea-water of a higher temperature, the gametes were either moribund or dead. At neither Woods Hole nor Naples have I ever observed filaments on mature echinid ova capable of fertilization. But stale eggs no longer capable of fertilization do produce them.

The indications are that this response of mature *Strongylocentrotus* eggs to insemination is due to the abnormal condition of the gametes. This position is supported by the observations of Mrs. Andrews which reveal that mature echinid eggs in an abnormal condition—produced by heat, pressure, or confinement—exhibit a cortical behavior markedly different from the normal. It is certain that Mrs. Andrews observed the production of filaments by *Echinus* eggs.

The literature affords no more beautiful observations on living eggs than those recorded by Mrs. Andrews. Her papers are marvels for their painstaking and accurate descriptions of the delicate and ever-changing cortical phenomena which echinoderm eggs exhibit throughout their development. Her meticulous account of the spinning processes which establish the hyaline plasma layer of echinid eggs after insemination, for example, has never been equalled; in comparison the descriptions by subsequent workers, who have completely ignored her work, are banal, superficial, and pitifully inadequate. It is most unfortunate that Mrs. Andrews' work has not received the recognition it so richly deserves.

Arbacia punctulata. Ovocytes of *Arbacia* when inseminated form processes which fifteen to twenty minutes after insemination are blunt papillæ much larger than the entrance cones found in normal fertilization. Wilson has described and photographed ovocytes of *Toxopneustes* showing these cones, as he named them. In addition, Seifriz has at some length described filaments on ovocytes of *Echinarachnius*. Runnström (1928) has given a review of the work on this phenomenon. Further comment is therefore unnecessary.

From the foregoing summary one is justified, I think, in reaching the conclusion, at least for the forms named, that cortical filaments as a response to insemination are produced only by eggs incapable of the fertilization-reaction: either the eggs, though in the fertilizable stage, are abnormal or they are immature. The question therefore arises concerning the production of filaments by eggs of *Asterias*. Is it possible that here also the phenomenon, as described by Chambers, is due not merely to the injury of the eggs incident to the micro-dissection methods which he employed, but also—and this is more cogent—to their abnormal condition? I believe that the evidence which I shall now present indicates that he did use abnormal eggs.

Asterias. In the first place, eggs of *Asterias forbesii* and *A. vulgaris* in the germinal vesicle stage produce cortical filaments on insemination. Such eggs never develop. Indeed, insemination, like treatment with butyric acid or heat (R. S. Lillie, 1914), inhibits maturation even. At Naples I found that eggs of *A.*

glacialis in the germinal vesicle stage react to insemination in a similar way. Hobson previously had noted the same phenomenon in *A. rubens*. Says Hobson: "Protoplasmic papillæ are found, so similar in their structure and behavior to those in *Echinarachnius* that, although these observations were made before I had seen Seifriz's paper, their repetition would simply duplicate his study." These eggs never complete their maturation or cleave, according to Hobson. He further suggests that this formation of protoplasmic papillæ "will be found to be widely spread in Echinoderms since the present writer has also observed similar formation of protoplasmic papillæ by unripe oocytes of *Holothuria nigra* in the presence of sperm."

In the second place, after the break-down of the germinal vesicle when the eggs are fertilizable or capable of responding to agents of experimental parthenogenesis, it is also possible to obtain cortical filaments as a consequence of insemination. In this case, however, the egg must be subjected to some form of injury: *e.g.*, pressure, overcrowding, CO_2, or increase in temperature. After complete maturation with the drop in fertilizability insemination likewise induces the formation of filaments.

(*a*) If eggs of *Asterias* which, after the rupture of the germinal vesicle, are capable of normal fertilization, maturation, cleavage, and embryo formation be inseminated under the pressure of a cover-slip, filaments are formed in abundance. For example, a very fine lot of eggs, taken from the ovaries of a shedding female, gave 99 per cent. cleavage, beautiful gastrulæ, and vigorous larvæ —in no wise to be distinguished from the larvæ produced from shed eggs. 0.2 cc. of these eggs inseminated, in finger bowls containing 200 cc. of sea-water, at ten minute intervals after removal from the ovaries to sea-water up to the time of the extrusion of the second polar body, showed no filaments. On the other hand samples of these same eggs inseminated under the pressure of a cover-slip did show filaments.

(*b*) Approximately 100 eggs were inseminated in ten cc. of sea-water at ten minute intervals after removal from the ovaries as in (a). Every egg cleaved normally. On the other hand, similar lots from the same ovaries each inseminated in a drop of water on a slide formed filaments and gave extremely low percentages of abnormal development.

(*c*) Eggs from one female were divided into two dense suspensions of 50 cc. each. *Lot A:* eggs were placed in 5 liters of sea-water. *Lot B:* eggs were placed in 150 cc. of sea-water. Thirty minutes later a drop of eggs from each lot was examined under the microscope: 90 per cent. of the eggs of *Lot A* were in process of maturation; 90 per cent. of the eggs of *Lot B* showed germinal vesicles intact. Eggs of *Lot A* on insemination showed no filaments, but those of *Lot B* whether with or without intact germinal vesicles did. 95 per cent. of the inseminated eggs of *Lot A* gave vigorous larvæ, while less than 1 per cent. of *Lot B* gave normal gastrulæ.

This observation is by no means an isolated one. Experience has shown that large volumes of sea-water are indispensable for the break-down of the germinal vesicle and therefore for optimum fertilization capacity as well as for experimental parthenogenesis. Eggs taken from the ovaries of a shedding female will yield a very low per cent. of maturation if crowded; the same eggs show close to 100 per cent. maturation in large dishes of sea-water. This point merits more than passing notice.

My first experience with the living egg of the starfish dates back to 1909. Repeated attempts to obtain viable larvæ from eggs inseminated after removal from the ovaries were failures. In June 1910, thanks to Dr. John W. Scott, who very generously gave me a male and a female, both of which were actively shedding, I was able to carry through my first lot of eggs to the brachiolaria stage. In 1911 I carried normally shed eggs inseminated with normally shed sperm through metamorphosis. Since then I have frequently obtained shedding animals, the eggs of which always gave a high per cent. of normal membrane separation, maturation, cleavage, and bipinnaria. These larvæ if fed metamorphosed. Indeed, during some years, notably June 1927, the ready shedding of the animals was actually a nuisance: animals procured in late afternoon often shed in the early morning hours and the eggs when removed from the tanks were found in cleavage stages.

Because of the extensive experimental work done on starfish eggs, it very early occurred to me that it might be of interest to learn whether normally shed eggs—as measured by the fertilization-reaction, by the rate, uniformity, and per cent. of cleavage

and by the size and viability of the larvæ, etc.—are superior to eggs obtained directly from the ovaries, according to the method which most workers employ. A moment's consideration suffices to show the significance of such a study: from it one might establish a basis for appraisal of all experimental work done on these eggs which fails to consider the normal development of normally shed eggs. Too generally, I think, workers have made experiments on starfish eggs without knowledge of the development of shed eggs.

Now the eggs shed by starfish are at once fertilizable. These eggs give practically 100 per cent. cleavage and beautiful vigorous larvæ of uniform size. I found that eggs taken from ovaries, which must stand in sea-water until breakdown of the germinal vesicle before insemination, are often inferior as revealed by the lower per cent. of cleavage and less viable larvæ of varying sizes. This seemed serious to me not only because it might cast suspicion on much of the experimental work done on such eggs but because it would seem to nullify my main object in using eggs of the starfish: namely, to study their fertilization in every stage of the maturation processes as a basis of comparison with those eggs in which the fertilization-moment is more rigidly fixed with respect to the stages in maturation. I soon found, however, that it is possible to obtain starfish eggs from the ovaries in every way equal to those that are naturally shed. The procedure is simple:

I either take the eggs from the ovaries of shedding females or, selecting the heaviest and most healthy-looking animals, remove the eggs from the ovaries at once. In either case I use at least three liters of sea-water in a dish of about 28 cm. in diameter to which I add either *one* ovary carefully exposed and removed or only the broad end of the ovary next the disc. After five minutes the ovary is removed. One drop of dry sperm is placed in 200 cc. of sea-water in which they are intensely active.

This method has given me most excellent results. On the other hand, eggs from unselected females concentrated in a small volume of sea-water, on insemination not only show cortical filaments, but also yield a small per cent. of abnormal cleavage and larvæ of low viability.

Fol, in his classic monograph on fertilization, emphasized the

importance of using fresh animals with distended ovaries. He also advised using large quantities of sea-water. In addition he cautioned, for both the sea-urchin and starfish, against contamination of the eggs by the cœlomic fluid.

(*d*) CO_2 and heat were each found capable of causing the production of filaments by eggs in the optimum fertilizable condition. If, for example, eggs, with the germinal vesicle broken down, which give 90 or a greater per cent. of membrane separation be inseminated after treatment for one minute with sea-water, charged with CO_2, they form filaments. Or, if such eggs taken from sea-water at 15°–16° C. be inseminated in sea-water at 26° C., they form beautiful filaments.

The foregoing brief account of the observations on eggs of the genus *Asterias* indicate (1) that in the germinal vesicle stage when incapable of fertilization they produce filaments as do immature eggs of *Thyone*, *Arbacia*, *Toxopneustes* (Wilson), *Echinarachnius* (Seifriz), *Holothuria nigra* (Hobson), *Asterias rubens* (Hobson); and (2) that in the fertilizable stage, after break-down of the germinal vesicle, if inseminated under adverse conditions, they produce filaments.

In the next place, after complete maturation the fertilizability of *Asterias* eggs falls off. During May and June of 1921, at Woods Hole, a careful study was made of this drop in the fertilizability of the eggs both normally shed and those taken from the ovaries. At the Naples Zoölogical Station this was found to be true for *A. glacialis* also. With the falling off in fertilizability, it was learned in 1926 and 1927, that the capacity of the eggs to produce filaments increases.

Observations on the production of filaments by completely maturated eggs as a response to insemination were among the first ones made. The eggs used fall into three classes: those normally shed, those from the ovaries of shedding females, and those from selected females with greatly distended ovaries. Of all three classes the eggs were in the optimum condition— as revealed on insemination, after break-down of the germinal vesicle, by the rate and quality of membrane separation, the regularity of cleavage, and the vigor of the larvæ. That is, eggs were inseminated at ten minute intervals after coming into sea-water throughout

the maturation process in order to fix the optimum moment for fertilization. With the completion of maturation, the inseminations were continued for three to ten hours longer, the production of filaments being noted at each insemination. No lot of eggs showing more than 10 per cent. intact germinal vesicles was ever used; nor was any observation made on eggs, samples of which gave less than 90 per cent. membrane separation. In other words, the maturated eggs that produced filaments were always from lots which, inseminated at some stage of maturation, gave optimum developments.

In August 1923, at Woods Hole, soon after the appearance of Chambers' paper on the mechanism of the entrance of the spermatozoön into the starfish egg, I was fortunate in collecting some very fine shedding *Asterias* which I used immediately. Again it was found that after maturation the fertilization capacity of the eggs is lost; with this loss, the production of filaments is increased. On the other hand, eggs inseminated as shed, with the germinal vesicle broken down, were immediately fertilizable—without filaments unless adversely treated—and gave beautiful development. On January 30, 1929, at the Naples Zoölogical Station I procured my first shedding *A. glacialis;* in Feburary also I had shedding females. These gave the same results obtained at Woods Hole.

Briefly, the observations indicate that maturated eggs lose capacity for normal fertilization and parallel with this loss runs the production of filaments. Three hours after the extrusion of the second polar body (*A. forbesii* and *A. vulgaris*) filament formation is first most readily noted. On *A. glacialis* (observations on eggs of four shedding females only) the time is about three and one-half hours. Until their death the eggs respond to insemination with the production of filaments.

Discussion.

If the observations given above be correct we may conclude that the production of filaments by *Asterias* ova, as well as of the other echinoderm ova studied, as a response to insemination is a phenomenon quite apart from the behavior in the normal fertilization-reaction. It follows, therefore, that any generalizations concerning its significance for the mechanism of sperm penetra-

tion into the animal egg must be tempered. Though the reasons for this statement seem fairly obvious, it may not be amiss to elaborate them somewhat. In addition, there is the primary need to consider specifically the case of the starfish egg. We may begin with Fol's original descriptions.

In his classic monograph on fertilization, Fol gave a detailed description of the entrance of the spermatozoön into the egg of *Asterias*. According to this account, several spermatozoa reach the outer border of the jelly hull; one gets through and moves toward the surface of the egg. The proximity of this spermatozoön causes the egg cortex to form a hyaline elevation, the *attraction cone*. The form and especially the length of the attraction cone vary, depending upon the rapidity with which the spermatozoön approaches the vitellus; if the approach be slow, the cone is prolonged as a filament whose length is half the diameter of the jelly hull; if the approach be rapid, the spermatozoön reaches the cone before it can elongate. The form of the cone thus depends upon the activity of that spermatozoön which first pierces the jelly hull (Fol, page 91).

As noted above, I have made observations on the penetration of the spermatozoön into the normal starfish egg in the optimum stage for fertilization. These observations have been supplemented by study of several series of sectioned eggs fixed at 5 or 10 second intervals beginning at 5 and ending at 120 to 180 seconds after insemination. In each series the eggs were from a single female and inseminations at 10 or 15 minute intervals from the time they were brought into sea-water until complete maturation; thereafter inseminations were made at 30 minute intervals for two or three hours. Thus, one series alone makes up over 200 stages of sectioned material. The rapidity of normal sperm attachment, due to the intense activity of the spermatozoa in optimum conditions, demands a study of fixed material despite the importance of observations on living eggs and the present-day vogue of disparaging the study of fixed cells.

On the basis of these observations I am forced to disagree with Fol's account of the formation of an attraction cone stimulated by proximity of the spermatozoön to the egg surface. As I see it, the phenomenon is as follows:

The intensely active spermatozoa rush toward the jelly hull; of these, one rapidly moving through it reaches the egg within 5 seconds after insemination. *The cone forms after the attachment of the spermatozoön to the vitelline membrane.* As the cone grows the spermatozoön—seen in both living and fixed preparations—is pushed off from the egg, a delicate strand connecting the tip with the apex of the cone. This strand never attains the length given by Fol, as half the diameter of the jelly hull, and of course could never therefore be equal to the greater length figured by Chambers. *Moreover, this strand is a prolongation of the spermatozoön, the tip of which is fixed within the cone.*

The point, which elsewhere (Lillie and Just) has been emphasized, is still considered the important one: namely, that before formation of the cone, the spermatozoön has been attached to the vitellus for about 45 seconds. The cone, whether blunt or elongated, is not, therefore, an attraction cone. Fol perhaps used too dense sperm suspensions which were above the level for optimum activity—since starfish spermatozoa are most highly active only in thin suspensions—though possessing fertilizing power and exhibiting increased activity when mixed with the eggs. But if he did this, it was not his greatest error. The main objection to his work lies in another direction.

Now Fol knew that eggs of *Asterias* are capable of insemination during first maturation. Such eggs subsequently show both polar bodies adhering to the vitellus separated from the membrane by a wide perivitelline space. They develop normally. Unfortunately Fol gave scant notice to this point, dismissing it with a few sentences. Instead, he focused his attention on the fertilization process in fully matured eggs. This meant, of course, that the eggs had been lying in sea-water for some time—about four hours at a temperature of 12° to 15° C. In my experience at least, after complete maturation, eggs of *Asterias glacialis* at Naples lose their capacity for normal fertilization; the character of the cleavages and the viability of the larvæ as well as the rate and quality of the fertilization-reaction reveal this. They therefore resemble eggs of the American species studied. Pressure, elevation of temperature, overcrowding—any one of these factors induces the formation of filaments. But we may here waive

them all: *Fol used stale eggs, i.e.*, eggs that had passed the optimum condition for fertilization and this fact alone accounts for his results on the penetration of the spermatozoön.

That Fol's observations are correct I do not for a moment dispute, nor do I deny Chambers' similar findings—I have too often seen the cortical phenomena which they described. What I do contend is that their account of the cone (or filament) formation belongs in the same category with those on immature unfertilizable eggs, fertilizable eggs rendered unfertilizable by brutal treatment, and stale eggs having passed the fertilizable period. If this contention be sustained, neither Fol's nor Chambers' work has any direct value for the normal fertilization-reaction *per se*. Nevertheless their work on abnormal eggs like that by others cited in this paper is of interest: it contributes additional testimony concerning the reactivity of the egg cortex.

There is available a sufficiently large body of evidence which indicates that the cortex (or ectoplasm) plays an important rôle in cellular activity. Whether the cells be amœbæ, egg cells, or tissue cells—*e.g.*, striated muscle or nerve of vertebrates—their surfaces are the seat of changes important for vital processes. These are not merely physico-chemical changes easily reducible to mathematical formulation which apply to non-living films of molecular dimensions. Rather, the cortex—I speak now especially of ova—is an easily visible structural entity of never-ceasing, well-defined changes. The reactivity of the cell as a whole—its individual and peculiar response to stimulation with attendant measurable physical and chemical changes—is largely, if indeed not wholly, a cortical (ectoplasmic) phenomenon. Cortical changes in ova are, therefore, no mere epiphenomena: they constitute the *sine qua non* of cellular life. In responding to and propagating the effects of the initial event in the fertilization-reaction, the attachment of the spermatozoön, the egg exhibits cortical changes wihch eventually modify the whole protoplasm and direct the course of ontogeny. The spinning activity of the cortex, to borrow Mrs. Andrews' happy phrase, is thus of great significance. Even that shown by eggs incapable of the normal fertilization-reaction, with which we are here concerned, has some value.

In the first place, these filaments formed by abnormal ova as a response to insemination are exaggerated entrance cones. Their formation is additional proof, if such be needed nowadays, that the penetration of the spermatozoön may be a phenomenon quite apart from fertilization *per se*. In the case of the immature egg, the production of filaments suggests that the mechanism for sperm entry is already laid down; in the case of the brutally treated normal, or of the stale, egg, that this mechanism persists. In all these three kinds of eggs the cortical response to insemination is diffuse and slow; it thus differs markedly from the sharply localized and rapid response of the normal egg in optimum fertilizable condition. Nor yet is the essential characteristic of the fertilization-reaction the formation of the sperm aster.

Years ago one thought of the essential phenomenon in fertilization as the introduction into the egg by the spermatozoön of centrosomes with attendant sperm aster formation. But Boveri showed—what subsequently Brachet has ponderously described, without, however, mentioning Boveri's work—that a spermatozoön may enter an echinid egg during maturation and form an aster. The egg, however, is not fertilized. It does not develop. Evidence indicates that sperm aster formation is due to the diffusion into the cytoplasm of material escaping from the broken-down germinal vesicle. Such asters form, for example, in eggs which sperm normally penetrate in the ovocyte stage when later this material is present in the endoplasm.

On the one hand, then, the egg lays down in its cortex the mechanism for sperm entry; on the other, it possesses aster-forming substances diffusing from the germinal vesicle into the cytoplasm. Neither is alone, nor are both together, sufficient for fertilization. There must be present in addition the fertilizin of Lillie at or in the cortex. Normal fertilization means the cortical reaction between spermatozoön and fertilizin which sets up the rapid explosive wave which, beginning at the point of sperm entry, sweeps over the egg surface.

In a more general way we must now consider the production of filaments by ova as a response to insemination.

Animal ova may be variously classified according to one or another of several criteria obtained through a study of their fertiliza-

tion processes. Such classifications are useful, as I have elsewhere pointed out (Just, '22), for an analysis of the fertilization-reaction; for with them we distinguish between the incidental phenomena and the sequelæ of the fertilization-reaction as well as the indicia of cell division, on the one hand; and the significant event in the fertilization-reaction *per se* on the other. Thus we may come to recognise the common factor in the fertilization process of all animal ova. Now one such classification of ova, with respect to their fertilization, we may make with sperm entry as the criterion: some ova possess micropyles, others do not; some form entrance cones, others do not. Also it has been held that some form attraction cones. The entrance cone, one understands of course, forms *after* the attachment of the spermatozoön to the egg surface. In my judgment, the evidence in favor of the formation of an attraction cone, *i.e.*, an elevation of the egg cortex *before* sperm attachment is extremely doubtful. If the observations here reported be correct, then the most generally accepted case, that of *Asterias* egg, is discredited. This too, we must conclude, in normal fertilization is an entrance cone.

This cone in *Asterias* egg closely resembles that in *Nereis* egg except that the duration of time between sperm attachment and its formation and between the latter and sperm engulfment are both much longer in *Nereis* egg. Moreover, the strand between the sperm head and the cone in both cases has the same origin, namely, from the spermatozoön itself. In echinid eggs, on the other hand, cone formation and sperm penetration are more rapid than in the starfish egg. Essentially, however, the process is the same in the three types. The retraction of the cone in the eggs of *Nereis* and of *Asterias* is very similar; whereas in echinid eggs the cone persists after the spermatozoön has traversed the cortex.

The fact that many ova do not possess cones renders it unlikely that their formation is an essential phenomenon for fertilization. Even if the mechanism of sperm entrance as described by Fol and by Chambers were correct, it would thus have only limited application for fertilization processes generally. And were it possessed of general application, it would, in view of the observations on the production of filaments by eggs of feeble or no fertilization capacity recorded above, give us little aid in the analysis of the

fertilization-reaction. The rapidity, the irreversibility, and the specificity of this reaction indicate a phenomenon common to all metazoan ova of far more fundamental significance than mere sperm entry. It is through the study of this common factor that we must hope to approximate more closely the solution of the fertilization problem.

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THE FERTILIZATION-REACTION IN EGGS OF *PARACENTROTUS* AND *ECHINUS*. [1]

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During three months of my stay at the Naples Zoölogical Station I had the opportunity, beginning January 25, 1929, of studying fertilization in the eggs of two echinids—*Paracentrotus lividus* and *Echinus microtuberulatus*. The work carried out on the eggs of these forms was largely a repetition of work previously done on the eggs of *Echinarachnius* and of *Arbacia*. Since certain of the results obtained on the eggs of these two American species have been used in support of Lillie's work, and since it has been suggested that some of the results are peculiar and not of general significance for the analysis of fertilization, it was felt that any data on European forms should be noted. The work was done most intensively during the month of February, following preliminary observations made during the last week in January. During March and April also observations were made. Observations were likewise made on the eggs of *Arbacia;* since the findings here were so closely similar to those obtained on the American species, no reference is made to them.

It gives me great pleasure to acknowledge my indebtedness to Prof. R. Dohrn., Director of the Naples Zoölogical Station, and to other members of the staff for unfailing courtesies which they extended me throughout my stay.

THE OBSERVATIONS.

The observations on the eggs of *Paracentrotus* and *Echinus* fall into three groups: first, observations on the rate of membrane separation; second, observations on the sperm agglutination capacity of the egg-sea-water; and third, observations on the inhibitory action of the perivisceral fluid.

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It will be convenient to record these observations on the eggs of *Paracentrotus* and *Echinus* in turn.

Paracentrotus.

Following insemination eggs of *Paracentrotus* show membrane separation beginning in 30 seconds. This was learned after repeated observations on the eggs from single individuals as long as the eggs remained viable after removal to sea-water. From January 25 to January 30 inclusive, for example, over a hundred records were made. These results were subsequently confirmed. Membrane separation in this egg is easily followed. Beginning at the point of sperm entry, it separates from the surface of the egg until finally entirely off; the membrane then becomes equidistant from the egg surface at all points. The cortex beneath the membrane is clearly defined, being made up of delicate papillæ which eventually are covered with a delicate membrane. Papillæ and membrane constitute the hyaline plasma layer. The fertilization cone which forms around the entering sperm is pronounced and easily visible. This fact makes it easy to mark the entrance point of the spermatozoön. In addition, eggs rapidly fixed at the moment that membrane separation begins show spermatozoa attached to the eggs in the zone of membrane separation.

Observations on the inhibitory action of perivisceral fluid reveal that in some cases inhibition may be complete. For example, lots of eggs from the same female whose eggs, thoroughly washed in sea-water, on insemination in sea-water had given 95 or a greater per cent. of fertilization, inseminated in the presence of perivisceral fluid showed no membrane separation or other signs of development. Such eggs in perivisceral fluid, however, after thorough washing in sea-water, provided the washing did not extend over six hours, were fully capable of fertilization. After about six hours in normal sea-water, fertilization capacity drops as shown not only by the slow rate of membrane separation but also by the small per cent. of cleavage.

Eggs of *Paracentrotus* allowed to stand in normal sea-water produce a sperm agglutinating substance. The power of this substance varies, depending upon the maturity of the ovaries.

The best results were obtained with eggs that were normally shed or with those from animals which exuded eggs when cut. Where eggs were taken directly from the ovaries, whether or not contaminated with perivisceral fluid, which might or might not inhibit fertilization, the agglutination tests were never so sharp.

Compared with *Echinarachnius* and *Arbacia* the egg of *Paracentrotus* is inferior to *Arbacia* for the study of inhibition to fertilization by the perivisceral fluid and for agglutination tests. This I attribute to the fact that one ripe *Arbacia punctulata* yields more eggs. With respect to membrane separation it is far superior to the American species of *Arbacia* in which the process is discerned with difficulty. In *Paracentrotus* it is remarkably clear. The reader will recall that Fol years ago observed the separation of the membrane in eggs of *Paracentrotus* beginning at the point of sperm entry. On the other hand, while the process of membrane separation is more rapid than that in *Echinarachnius* the egg is a better object for study because it is more hardy.

Echinus.

In eggs of *Echinus* the first indication of membrane separation is a wrinkling of its surface. This takes place before actual separation begins. The longest time between insemination and the beginning of membrane separation noted was 100 seconds; the shortest time, 45 seconds. The average time for all of the experiments was between 45 and 50 seconds. In general the speed of separation may be correlated with fertilization capacity. For example, whenever membrane separation was delayed for 100 seconds about 50 per cent. of the eggs showed no membrane separation whatsoever. In one such lot of slowly reacting eggs, which in seven trials gave the time between insemination and membrane separation between 95 and 100 seconds, heavy insemination induced membrane separation only after 180 seconds. The membrane is completely off and equidistant from the egg at all points 150 seconds after insemination. In this egg vesicles comparable to those noted in *Echinarachnius* were found in the perivitelline space. The cortex under the membrane is well marked. I may note in passing that eggs in the germinal vesicle

stage whenever found always responded to insemination by forming papillæ (C. F. Runnström, 1924 for *Psammechinus miliaris*).

The presence of perivisceral fluid strongly inhibits fertilization. When the animals are used soon after collection their eggs give remarkably uniform results with respect to membrane separation, cleavage, and later development. I was frequently able to keep plutei in the laboratory for two weeks after insemination. The inhibition to fertilization tested as previously in the work on other forms (Lillie, '14, Just, '22, '23) could not therefore be attributed to the quality of the gametes.

Uninseminated *Echinus* eggs in sea-water produce a sperm agglutinin. The production of the agglutinin runs parallel with fertilization capacity. Of two lots of eggs, for example, from two different females, one producing strong agglutinating substance and the other a weak, membrane separation, per cent. of cleavage, and normality of later development are superior in the former. On February 21, observations were made on the eggs from ten females. In each case high agglutinin production was correlated with optimum fertilization capacity. Throughout the course of the observations eggs of low agglutinin output gave poor response to insemination.

Discussion.

Fol years ago noted the fact that following insemination the vitelline membrane begins to separate from the surface of the egg of *Strongylocentrotus* (*Paracentrotus*) at the point of sperm entry. This is likewise true for eggs of *Echinarachnius* (Just, '19) and *Arbacia* (Just, '28). The observations here reported thus confirm earlier ones. The egg of *Arbacia* is the most difficult object for the observation of this phenomenon; nevertheless study of both the living and the sectioned egg establishes the fact. On the other hand, there is no difficulty whatsoever in following the sequence of events between insemination and membrane separation if one employs any of the three other species named. Any worker possessing only mediocre powers of observation, therefore, should be able to prove to his own satisfaction that eggs, of these three forms at least, separate membranes, beginning at the point of sperm entry. This is a question not of argument or "interpre-

tation"; rather, it is merely a matter of being able to make a simple observation.

The appearance of vesicles in the perivitelline space is worthy of note. As the membrane separates from the echinid ova studied—except those of *Arbacia*—the cortical break-down, which is responsible for membrane separation, is easily visible under low power of the microscope. Progressively with the separation of the membrane, vesicles appear in the perivitelline space. They gradually disappear as they move across the space from the vitellus toward the membrane. I have repeatedly demonstrated this phenomenon as it occurs in eggs of *Echinarachnius*; many workers, however, though they have observed it have been sceptical of its being a normal process. It was interesting to find that the Naples echinid ova exhibited the same phenomenon.

Concerning the rôle of perivisceral fluid as an inhibitor for fertilization, I need say very little. Here again, Fol pointed out this fact. Other data indicate that such "blood" inhibition is by no means confined to the echinoderms.

Similarly, since some of the earlier work on the agglutination of spermatozoa by specific egg-sea-water was done on European echinids, this topic may be dismissed with a word. My observations at Naples got a long way toward strengthening the position that fertilization capacity and sperm agglutinin production run parallel. Stale eggs from lots which previously had given the optimum fertilization-reaction either gave low or no response to insemination. The evidence from the observations makes it highly improbable that such eggs failed to fertilize because thev were dead.

It would be hazardous to assert that the fertilizin theory based on the reactions between spermatozoa and ova and between spermatozoa and egg-sea-water with and without perivisceral fluid of the few forms studied is capable of application to fertilization in all metazoa. For these particular forms, however, the theory still holds.

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